University of Alberta

Hypoglycemic and hypolipidemic effects of galactomannan from fenugreek (*Trigonella foenum-graecum* L.) grown in Canada

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Spring 2007

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ISBN: 978-0-494-29753-7
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ISBN: 978-0-494-29753-7

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To my father and mother

Abstract

Fenugreek (*Trigonella foenum-graecum* L.), a legume grown in the Middle East and Asia, has thus been shown to be beneficial in the treatment of diabetes. In recent years, fenugreek has become a forage crop in the western Canadian Prairies. However, the chemical composition and health effects of Canadian fenugreek have not been established. The present study was undertaken to a) carry out a proximate analysis of the seeds of a variety of fenugreek lines, grown in Southern Alberta, b) extract and purify galactomannan, a soluble fiber, and c) examine the effects of feeding the purified galactomannan on blood glucose and lipids in rats. Compared to an Asian grown fenugreek line, the Canadian grown lines (F-70, F-86, L-3314, and Amber) were found to contain approximately 10% more galactomannan. A method was developed to extract galactomannan from the Amber line. This method involved fractionation of ground seeds into a fraction of seed coat/aleurone/endosperm, followed by precipitation of the galactomannan with ethanol to yield a 91.4% pure extract. Galactomannan was highly viscous and the viscosity was not affected by the presence of 10% or 20% sucrose in a solution. Using JCR rats (model animals for insulin resistance), an *in vitro* study revealed that the presence of the galactomannan extract in the intestine, markedly diminished intestinal uptake; this effect did not differ between lean and obese rats. Feeding normal Sprague-Dawley rats with a high sucrose diet containing galactomannan (2.5% or 5.0% w/w) for 4 weeks resulted in reductions in plasma levels of total triglycerides, total cholesterol, LDL-cholesterol, and epididymal adipose tissue weight, compared with those of the animals fed the control diet ($p < 0.05$). It was also noteworthy

that galactomannan group (5.0% w/w) displayed a decreased insulin response during glucose tolerance test ($p < 0.001$), suggesting improved insulin sensitivity. However, lower body weight gain ($p<0.05$) in parallel with less food intake ($p<0.05$) were found in 5.0% (w/w) galactomannan-fed rats compared to the control. In conclusion, feeding galactomannan extracted from Canadian-grown fenugreek has the potential to modify both glycemic and lipidemic status in Sprague-Dawley rats.

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Dr. Tapan Basu, for his continuous guidance, and support throughout my Ph.D. program. I would also like to acknowledge my supervisory committee members: Dr. Thavaratnam Vasanthan and Dr. Surya Acharya for their advice on the project, and Dr. Alan Thomson for providing invaluable guidance and support throughout the process of conducting my experiments. I would also like to extend my gratitude to Dr. Catherine Field for her constructive suggestions during my research work. I am appreciative of Dr. Linda McCargar for serving on my candidacy examination committee. I would also like to thank Dr. Sukhinder Kaur for acting as my external examiner. In addition, I would like to thank Dr. Buncha Ooraikul for facilitating my coming to the University and for his valuable guidance to my program, Dr. Feral Temelli for the opportunity to work in her laboratory, Dr. Peter Sporns and Dr. Patrick Nation for their valuable suggestions.

I sincerely appreciate and acknowledge the contribution of Dr. Takuo Nakano, Dr. Yeow Goh, Dr. Jihong Li, Dr. Wettasinghe Mahinda, Dr. Laurie Drozdowski, Dr. Yumiko Hoyano, Ms. Julie Laverdiere, Mr. Jun Gao, Dr. Zvonko Burkus, Mr. Paul Moquin, Mr. Bernhard Seifried, Mrs. Donna Taylor, and Mrs. Elisa King. I truly appreciate the friendship developed during my study with Dr. Min Chen, Mrs. Lisa Sun, Dr. Abdul Faraj, Ms. Minh Huynh, Ms. Judy Yeung, and Mr. Jacob Atakora.

Special acknowledgement is owned by my parents and brothers for their love, faith, patience, and support, to help me achieve my goals.

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List of Abbreviations

4-OH-Ile	4-Hydroxyisoleucine
ACTH	Adrenocorticotropic hormone
AGE	Advanced glycosylation end product
ANOVA	Analysis of variance
BBM	Brush border membrane
BLM	Basolateral membrane
CCK	Cholecystokinin
CHD	Coronary heart disease
CVD	Cardiovascular disease
DM	Diabetes mellitus
DMH	Dimethyhydrazine
FAO	Food and agriculture organization
FAPWC	Faculty of Agricuture, Forestry and Home Economics' Animal Policy and Welfare Committee
FFA	Free fatty acids
G'	Elastic modulus
G"	Loss modulus
G:M	Galactose:Mannose
GAL	Galactomannan
GC	Gas chromatography
GIT	Gastrointestinal tract
GLUT 2	Glucose transporter 2
GLUT 4	Glucose transporter 4
GLP-1	Glucagon-like Peptide 1
HCl	Hydrochloric acid
HDL	High density lipoprotein
HMGCoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPLC	High performance liquid chromatography
HSL	Hormone sensitive lipase
IGT	Impaired glucose tolerance test
JCR	James C. Russell
Km*	Apparent Michaelis constant
LBG	Locust bean gum
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
MW	molecular weight
min	minute
NaOH	Sodium hydroxide
NEFA	Non-esterified free fatty acid
ObR	Leptin receptor R
OGTT	Oral glucose tolerance test
PAP	Phosphadic acid phosphatase activity
RO	Reverse osmosis

RT	Room temperature
SCFA	Short chain fatty acid
SEM	Scanning electron microscopy
SGLT-1	Sodium-dependent glucose cotransporter1
SHROB	Obese spontaneously hypertensive rat Koletsky strain
SRD	Sucrose-rich diet
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TBARS	Thiobarbituric acid reactive substances
TEAA	Total essential amino acid
TG	Triglyceride
TNF	Tumor necrosis factor
VLDL	Very low density lipoprotein
UWL	Unstirred water layer
Vmax	Maximal transport rate
WHO	World health organization
w/w	weight for weight basis
w/v	weight for volume basis

Chapter 1

Introduction

Legumes, belonging to the family Leguminosae, have long been recognized to be functional foods. In comparison to the more commonly eaten grain products, however, the health benefits of legumes have been overlooked. Legumes have a low glycemic index (Bornet *et al.*, 1989), and they have been shown to have hypocholesterolemic effects (Anderson and Chen, 1997). It is increasingly recognized that the physiological effects of different legumes vary significantly. These differences may have resulted from variations in type and quantity of polysaccharides, protein make-up, and variability in phytochemical content. It seems possible that investigation of less commonly consumed legumes may reveal new sources of functional foods, which will have the potential to prevent and treat chronic diseases. One of these legumes has been identified as fenugreek, which has been used for centuries in folk medicine to treat for a variety of diseases, ranging from diabetes to cancer (Max, 1992).

Fenugreek (*Trigonella foenum-graecum* L.) is native to the Mediterranean region, Central Asia and North Africa. The use of this legume in various countries is reflected by its various names: 'Methi' in India, 'Pazhitnuk' in Russia, and 'Helbeh' in the Mediterranean countries. It is an annual plant; its ideal growing conditions that include tolerant 10-15 °C of frost, soil pH: 5.4-8.0, and annual precipitation: 25-150 cm (Duke, 1986).

Fenugreek seeds are popularly used as an ingredient in spice blends, especially in Asia and Africa. The seeds are about 0.5 cm in diameter and are irregularly shaped, very

hard, and tan or mustard in color. The flavor of fenugreek is similar to maple syrup although it has a strong bitter taste. Its seeds have a long history of medicinal uses for a wide range of pathological conditions (Fazli and Hardman, 1968; Khosla *et al.*, 1995). The latter include bronchitis, prostatitis, lumbar pain, and kidney ailments (Max, 1992). More recently, fenugreek has been recognized to have hypoglycemic and hypocholesterolemic properties and hence the therapeutic potentials in diabetes and coronary heart disease (Zia, 2001; Mondal *et al.*, 2004).

Fenugreek seeds contain 45-60% (w/w) carbohydrates (mainly galactomannan), 6-10% (w/w) lipids (mainly polyunsaturated fatty acids), and 20-30% (w/w) protein (rich in methionine, arginine, alanine, glycine, but poor in lysine). The 4-hydroxyisoleucine has been found to be a major free amino acid in the seeds. The seeds contain 5-6% (w/w) saponins and 2-3% (w/w) alkaloid (Rao *et al.*, 1996).

The fenugreek seeds have been identified to contain three potential biologically active components, which include galactomannan (a soluble fiber), steroidal saponins, and 4-hydroxyisoleucine (an amino acid). These components are thought to be effective in lowering blood glucose and lipid levels (Valette *et al.*, 1984; Ribes *et al.*, 1987; Sauvaire *et al.*, 1998; Zia, 2001).

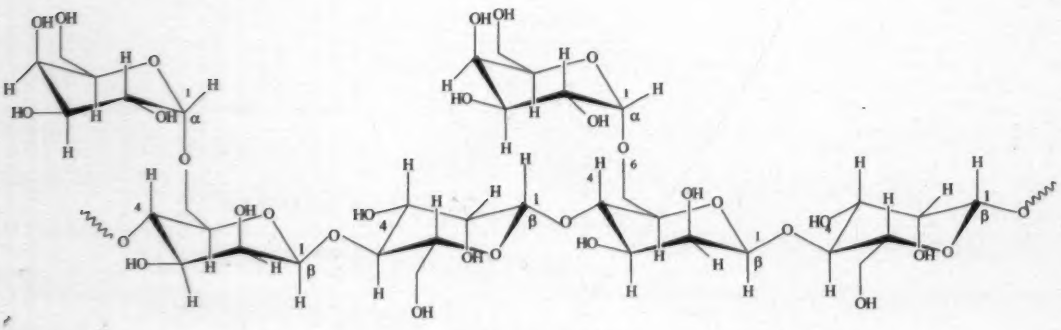
Galactomannans

Galactomannans are a group of hydrocolloids (gums) that are widely used in the food industry as thickeners, binders, stabilizers, emulsifiers, and suspending or gelling agents; they are functional, available, and low in cost. Their function and physical

properties, including solubility, gelling behavior and viscosity, are related to the molecule structure, sugar composition, degree and distribution of branching and polymerization.

The seeds of leguminous plants have mucilaginous endosperm, which contain galactomannans. The latter represent polymers of galactose and mannose, consisting of a backbone of (1→ 4) linked β-D-mannopyranosyl units with chains of (1→ 6) α-D-galactopyranosyl groups (Fig 1-1). Guar gum, locust bean gum (LBG), tara gum, and fenugreek gum are galactomannans that have different galactose:mannose (G:M) ratios and distributions of galactopyranosyl units along the mannan chains. The variability in galactose composition and distribution along the mannan main chain are responsible for variations in solubility and rheology of different sources (Maier *et al.*, 1993; Garti *et al.*, 1997). The solubility and viscosity are increased when the G:M ratio increases from 1:4 in LBG to 1:3 in tara gum or to 1:2 in guar gum.

Fig 1-1. Chemical structure of galactomannan



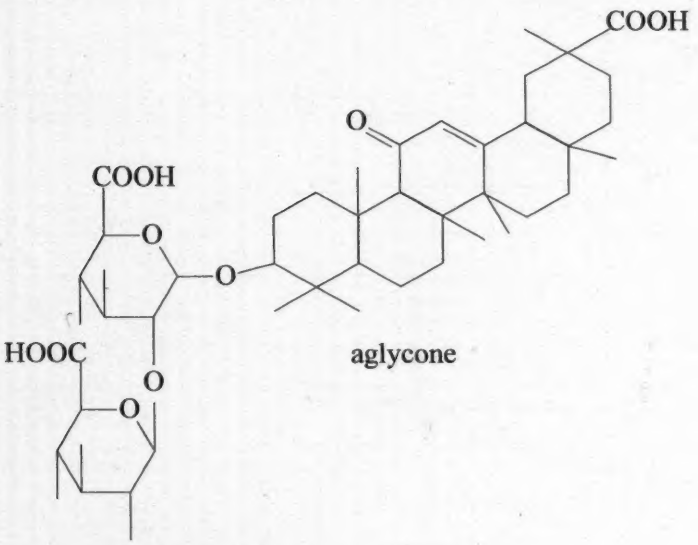
Fenugreek galactomannans are hydrophilic molecules. They have numerous free hydroxyl-groups, which can form hydrogen bonds with water. The latter is held within the polysaccharide matrix, unable to flow away, and the system has the semisolid properties with the characteristic of a gel (Oakenfull, 1996). In fenugreek galactomannan, the substitution rate of G:M is 1:1, making it superior in terms of its gel-forming characteristic over other galactomannans (Evan *et al.*, 1992).

Steroidal Saponins

Saponins are naturally occurring glycoside compounds found in a wide variety of food, forage plants, and to a lesser extent in marine animals. Fenugreek seed contains at least a dozen different saponins (Sauvaire *et al.*, 1996). These saponins mainly consist of an aglycone (e.g., sapogenin, sapogenol) linked to one or more sugars (Fig 1-2). The sapogenin (a bitter part) is hydrophobic, and the sugar part is hydrophilic. These structures give saponins their characteristic surface activity to form oil-in-water emulsions, and act as protective colloids. The structure also provides the ability to bind strongly to other components of the plant matrix.

Saponins have been shown to be excellent emulsifiers, foam stabilizers and cream mixer. They are widely used as a foaming agent in many products such as root beers, confectioneries, baked goods, cosmetics, shampoos, emetics, and cough syrups to facilitate expectoration. Saponins are used in animals for the purposes of increasing feed intake and growth rate, and accelerating the body's ability to absorb calcium and silicon (Fenwick *et al.*, 1991). Diosgenin of fenugreek seeds is widely used as a starting material for sex hormones in the pharmaceutical industry (Oakenfull and Sidhu, 1990).

Fig 1-2 Chemical structure of steroidal saponins



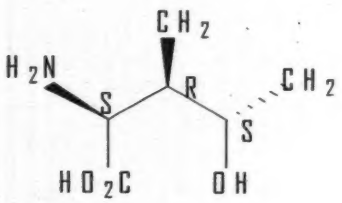
2 molecules of glucoses

4-Hydroxyisoleucine

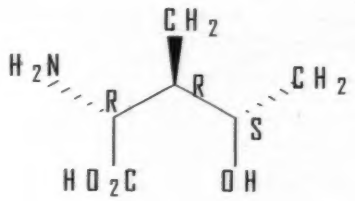
4-Hydroxyisoleucine (4-OH-Ile) is a polar amino acid that was firstly isolated and identified by Fowden *et al.* (1973). Studies have shown that 4-OH-Ile represents up to 80% of free amino acids in fenugreek seeds, but is absent from the seed reserve proteins. Fowden *et al.* (1973) obtained evidence of a relationship between isoleucine and hydroxyisoleucine, but the nature of the enzymatic system involved was not fully established. Among the various oxygenases present in plant tissues, cytochrome P450 has been suggested as a potential candidate for catalysing hydroxylation of isoleucine into 4-OH-Ile.

There are two diastereoisomers of 4-OH-Ile in fenugreek seeds (Fig 1-3). The major one has been found to have 2S, 3R, 4S configuration and represents up to 90% of total 4-OH-Ile content of the seeds. The minor possesses 2R, 3R, 4S configuration. The major isomer has been found to be an insulinotropic compound in both *in vitro* and *in vivo* studies (Sauvaire *et al.*, 1998; Broca *et al.*, 1999). Although there have been no large clinical trials performed, this potential health property has led to it being used by some as a nutraceutical. 4-OH-Ile is used in artificial flavorings such as vanilla, caramel, maple syrup. 4-OH-Ile is highly unstable and once exposed to air and moisture, it starts to degrade with characteristics strong odor. Thus, for long shelf life stability, it is important to store it under nitrogen pressure (Narender *et al.*, 2006).

Fig 1-3 Chemical structure of 4-Hydroxyisoleucine



Major isomer (2S, 3R, 4S)



Minor isomer (2R, 3R, 4S)

HEALTH POTENTIAL BENEFITS OF FENUGREEK SEEDS

1.1 Antidiabetic activity

Some isolated studies conducted on human subjects have shown that fenugreek reduces the postprandial glucose response in both non-diabetic (Sharma, 1986) and diabetic subjects (Mardar, 1988; Sharma and Raghuram, 1990). A gum isolated from whole and defatted fenugreek seeds had been found to reduce area under the glucose curve in parallel with a reduction in postprandial insulin response; in contrast, degummed seeds were reported to not influence the glycemic response (Sharma, 1986). The effect of postprandial glucose level was observed in subjects with type 2 diabetes (T2D) when 15 g of ground fenugreek seeds were added to a meal tolerance test (Madar *et al.*, 1988). These results were further confirmed in long-term metabolic studies (Sharma and Raghuram, 1990; Sharma *et al.*, 1990). In a study of Sharma *et al.* (1996), a prescribed diet (protein:fat:carbohydrate 20:20:60) without and with fenugreek seed powder (25 g/d) was given to 60 T2D subjects for 24 weeks. Results of this study showed that fenugreek seed-containing diet reduced fasting blood glucose and improved glucose tolerance in subjects.

Animal studies have been carried out to investigate the mechanisms by which fenugreek induces a hypoglycemic effect. Using streptozotocin-induced diabetic rats, gavage of ground fenugreek seeds in combination with a standard starch solution had been shown to decrease markedly the postprandial glucose tolerance curves (Madar, 1984). These results have been supported by Khosla *et al.* (1995), who had shown that feeding a diet containing fenugreek seeds (2-8 g/kg) for 30 days resulted in a significant

decrease in blood glucose level in both non-diabetic controls and alloxan-induced diabetic rats; the hypoglycemic response was dose related.

A reduced rate of gastric emptying is thought to play an important role for the fenugreek-associated decrease in glycemic response. The magnitude of the hypoglycemic effect of fenugreek was thus found to be greater when administered 15 min prior to a meal than if it was given with the meal (Sharma, 1986). The high viscous gel fraction of fenugreek, rich in galactomannans, is believed to be an effective hypoglycemic agent (Madar and Shomer, 1990).

Galactomannan

Although the beneficial effect of fenugreek seed has been known for several years, the attention to its active principle was only recent. Earlier, the hypoglycemic effect of fenugreek was attributed to its major alkaloid content (i.e. trigonelline) (Mishkinsky *et al.*, 1967). However, studies conducted involving the administration of different amounts of the alkaloid present in fenugreek to diabetic patients did not show significant hypoglycemic activity (Raguhuram *et al.*, 1994) Recently, there is evidence to show that the hypoglycemic effect of fenugreek is attributable to the fiber (mainly galactomannan), which constitutes as much as 40-50% (w/w) of fenugreek seeds. Gum isolated from fenugreek seeds when fed to normal subjects significantly lowered glucose response at 30 and 60 minutes after an oral glucose load (Sharma, 1986). These effects have been observed in other studies with T2D subjects (Puri *et al.*, 2002).

A dose response of fenugreek galactomannan had been studied in healthy subjects (Wolever, 2002). Given a glucose solution with different doses (1, 2, 4 or 8 g) of

fenugreek galactomannan extract, glycemic response was lowered with all doses in a linear dose-response fashion. However, the effect only reached to a significant level with 8 g of extract in a meal compared to control containing no extract. These effects were further confirmed in longer metabolic study for 8 weeks in T2D (Abe, 2002). It was shown that 4 g of extract divided into three doses per day, reduced blood glucose levels compared to placebo. These subjects were then given a reduced dose of extract (2 g) for the next 4 weeks. The latter was shown to maintain the same beneficial effects on glycemia in the T2D subjects as the higher dose. The gel-like property of extract in the stomach and its effect on slowing glucose absorption and satiety is believed to be a mechanism of these results (Abe, 2002).

Blood glucose lowering effect has been consistently observed with galactomannan extracted from a different source (i.e. guar) (Evan *et al.*, 1992). Several hypotheses have been proposed to explain its action within the small intestine. In glucose load studies, it seems to depend mainly on the capacity of the polymer to increase the viscosity of gastric and upper intestinal content (Cherbut *et al.*, 1990; Ellis *et al.*, 1996; Edwards *et al.*, 1998). The increase in viscosity can affect gastric function (e.g. emptying) and inhibit propulsive and mixing effects generated by peristalsis (Blackburn *et al.*, 1984; Read and Eastwood, 1992). In long term feeding, the viscosity formed by galactomannan in the stomach can create a feeling of satiety, promoting decreased food intake after a meal (Ludwig, 2000).

Although the galactomannan is likely a major factor, researchers believe that it is not the only component in fenugreek with hypoglycemic effect. An amino acid unique to

fenugreek seeds, 4-hydroxyisoleucine, has been shown to improve blood glucose levels when fed to subjects with T2D (Broca *et al.*, 1999; 2000).

4-Hydroxyisoleucine

The amino acid 4-OH-Ile found in fenugreek has both insulinotropic and antidiabetic properties (Broca *et al.*, 1999; 2000). Animal studies have been carried out to investigate the mechanisms by which 4-OH-Ile induces a hypoglycemic effect. Using intravenous and oral glucose tolerance in Streptozotocin-induced diabetic rats had been shown that the administration of 4-OH-Ile (50 mg/kg in 1 ml saline intraperitoneal injection) for 5 days improved glucose tolerance and partially restored the glucose-induced insulin response (Broca *et al.*, 1999). To investigate the mechanisms of this 4-OH-Ile, an i*n vitro* study was carried out by the same investigators indicating that 4-OH-Ile directly stimulated pancreatic β cell insulin release. This stimulation was seen in the micromolar range, but only in the major isoform of the amino acid (Broca *et al.*, 2000).

4-Hydroxyisoleucine increased glucose-mediated insulin release, through a direct effect on isolated islets of Langerhans from both rats and humans (Sauvaire *et al.*, 1998). The stimulating effect of 4-OH-Ile was strictly dose dependent of glucose. It is ineffective at low (3 mmol/l) or basal (5 mmol/l) glucose concentrations. However, at moderate (8.3 mmol/l) to high (16.7 mmol/l) concentrations of glucose, 4-OH-Ile potentiated the insulin secretion (Sauvaire *et al.*, 1998). This secretagogue (4-OH-Ile) could therefore be considered as a potential treatment of T2D.

The effect of 4-OH-Ile extracted from fenugreek seed on the rate of muscle glycogen resynthesis was investigated. The study was conducted by using 4-OH-Ile mixed with dextrose solution (1.8 g/kgBW) given to trained non-diabetic male cyclists compared to dextrose solution alone (Ruby, 2005). The result showed that 4-OH-Ile mixed with dextrose promoted a more rapid rate of glycogen resynthesis (63% higher) following glycogen depleting cycle exercise. Plasma insulin was not altered in this study. It was hypothesized that 4-OH-Ile may increase the rate of muscle glycogen resynthesis via enhanced insulin receptor activity or amplified glucose transporter (GLUT4) translocation independent of the traditionally proposed contractile and insulin regulated processes (Ruby, 2005).

1.2 Modifying effects on lipid metabolism

Studies carried out in human subjects have shown that fenugreek reduced plasma cholesterol levels in both healthy and normohypocholesterolemic-T2D subjects (Sharma *et al.*, 1990; 1996). Defatted fenugreek seeds reduced total cholesterol and LDL cholesterol levels; the level of HDL cholesterol, however, remained unchanged. Similar results were observed in hypercholesterolemic-T2D subjects when 100 g of ground fenugreek seeds were divided into two doses each day and added to meal for 10 days (Sharma and Raghuram, 1990).

Results from animal studies helped to elucidate potential mechanisms by which fenugreek acts as a hypocholesterolemic agent. Ground fenugreek seeds, added to a hypercholesterolemia-inducing diet in rats, prevented elevation of plasma cholesterol levels by increasing fecal bile acid and cholesterol excretion (Sharma, 1984, Basch *et al.*,

2003). When various fenugreek fractions were tested, it was shown that gum isolates (dietary fibers) and crude saponin extracts play important roles for the hypocholesterolemic characteristics of the whole fenugreek seeds (Sharma, 1986a). The possible mechanism of these fractions will be explained below.

Galactomannan

Fenugreek gum significantly reduced plasma cholesterol levels. When feeding a hypercholesterolemia-inducing diet to rats, it was observed that adding galactomannans from fenugreek lowered both liver and plasma cholesterol levels and the rate of hepatic synthesis of cholesterol was significantly decreased (Evans *et al.*, 1992). However, in this study, it is quite possible that both the dietary fiber and saponins-rich fraction contribute to the cholesterol-lowering capabilities of whole fenugreek seeds. Later, a 60 double-blind, placebo-controlled study with T2D with hypercholesterolemic adults showed a significant decrease in levels of total cholesterol (by 14 % from the control), low density lipoprotein (LDL, by 16 % from the control) in patients receiving fiber fraction of fenugreek (Sowmya and Rajyalakshmi, 1999).

The fiber fraction in fenugreek seeds has been shown to bring about a hypocholesterolemic effect through different mechanisms. One mechanism could be that soluble fiber increases the viscosity of the digested chime (Spiller, 1999). A second possibility arises from an increase in the thickness of the unstirred water layer (UWL) in the small intestine thereby inhibiting the uptake of cholesterol and bile acids (Vahouny, 1982; Spiller, 1999). A third possible explanation suggests that fiber reduces the rate of diffusion of cholesterol toward the absorptive mucosal surface (Jenkins *et al.*, 2000).

It was proposed that galactomannan in fiber fraction lowers lipid absorption through impaired lipid emulsification. This is due to increased lipid droplet size in micelle, attributing to decreased surface area for lipolysis (Ribes *et al.*, 1986). The network formation of galactomannan traps bile acids in the intestinal lumen, resulting in increased fecal bile acids and neutral sterols losses, thus reducing the quantities of bile acids recycled to the liver. The loss of bile acids is compensated by the enhanced conversion of cholesterol to bile acid in the liver, resulting in reduction of cholesterol stores in the liver (Jenkins *et al.*, 2000).

Another possible mechanism is that the fiber fraction in fenugreek suppresses hepatic cholesterol synthesis. Soluble fiber is fermented in the colon by the microflora and as a result produces volatile fatty acids (i.e. propionate) that are released into the blood stream. Propionate inhibits 3-hydroxy-3-methylglutaryl-coenzymeA (HMG CoA) reductase activity, thus reducing hepatic cholesterol biosynthesis (Jenkins *et al.*, 2000; McCarty, 2002).

The relationship between structure and physiological function of galactomannan has been studied among three different sources: fenugreek gum, guar gum, and LBG. When included in the diets of cholesterol-fed rats (10g/Kg), all three gums significantly lowered plasma cholesterol compared with a control diet based on purified cellulose. The cholesterol-lowering effect was consistent and in the magnitude of fenugreek > guar > LBG (Evan *et al.*, 1992). This result indicates that the cholesterol lowering activity of galactomannan depends on the G:M ratio.

Saponins

There are several possible mechanisms by which saponins might affect cholesterol metabolism. The most obvious and of longest standing is that saponins can form complexes with cholesterol in the intestinal lumen and thus inhibit its absorption (Gestetner *et al.*, 1972; Fenwick *et al.*, 1991; Petit *et al.*, 1995). Administration of steroid saponins mixed with food led to a decrease in total plasma cholesterol in normal and streptozotocin-induced diabetic rats (Sauvaire *et al.*, 1996). As for plasma lipoproteins, the decrease in VLDL-, LDL-cholesterol without any change in plasma free cholesterol indicated a reduction in cholesterol esters. The absence of any significant modification in triglycerides suggested a decrease in the LDL concentration by saponin and/or sapogenin treatments. Moreover, the results showed that approximately 60% of saponins are hydrolyzed into sapogenins in the digestive tract. These aglycones may be implicated together with the parent saponins in the inhibition of cholesterol absorption (Oakenfull and Sidhu, 1990; Sauvaire *et al.*, 1996).

Another possible mechanism is that the steroid aglycone part of saponins binds strongly with bile acids making the bile salts unavailable to bind with cholesterol. This is demonstrated in studies that used saponins from different plant species such as alfalfa, quillaja soya beans, navy beans, chick peas, and fenugreek. In these studies, it was shown that saponins interact with sterols in the gastrointestinal tract (GIT) in a way which might prove beneficial to humans (Milgate and Roberts, 1995).

Using an ethanol extract from ground fenugreek seeds, it was found that the saponin-rich isolate lowered plasma cholesterol levels in hypercholesterolemic rats by 18–26% (Stark and Madar, 1993). The ethanol extract inhibited bile acid absorption of

inverted segments of rat intestines in a dose-dependent manner. This suggests that an interaction may be occurring in the digestive tract between bile acids and saponins. It has been hypothesized that micelles are formed from bile acids and saponins, which are not available for absorption due to their large molecular size (Sidhu and Oakenfull, 1986)

Apart from binding, and hence limiting the absorption of dietary cholesterol or bile acids, saponins have the capacity to alter cholesterol metabolism by interfering with the enterohepatic circulation of bile acids. Non-absorbable bile acids are passed on into the colon and eventually excreted. This loss by fecal excretion is compensated by increased synthesis from endogenous cholesterol, resulting in lower plasma and liver cholesterol levels (Heaton, 1972; Oakenfull and Sidhu, 1990)

Results in human trials however are conflicting. Initial experiments using saponins showed increased fecal excretion of bile acids and neutral sterols, but no effect on plasma cholesterol was evident (Potter *et al.*, 1980). This result may be due to the low saponin intake (approximately 500 mg/day). A follow up trial by Calvert *et al.*(1981), using hypercholesterolemic subjects did not produce any effect on plasma cholesterol or excretion. Overall, the work done on saponins confirms its anti-hypercholesterolemic activity in animals. Comparisons are difficult between trials due to the use of different types of saponins.

1.3 Other therapeutic potentials

Antioxidant activity

Fenugreek seeds are rich in flavonoids (e.g., Apigenin, luteolin, orientin, vitexin, quercetin). Some flavonoids are reported to possess antioxidant properties and have been

shown to be potent inhibitors of LDL oxidation, platelet aggregation and adhesion (Kaviarasan *et al.*, 2004). The aqueous extract of fenugreek seeds reduced lipid peroxidation by inhibiting the production of thiobarbituric acid reactive substances (TBARS) (Howard and Kritchevsky, 1997). This effect of fenugreek aqueous extract is comparable with α-tocopherol and glutathione (Thirunavukkarasu, *et al.*, 2003).

Anti-carcinogen activity

Fenugreek seeds possess anti-carcinogenic properties due to the inhibition of β -glucuronidase. In a study by Devasena and Menon (2003), rats were fed with fenugreek seed powder at a dose of 2 g/kg body weight along with subcutaneous injection of 1,2-dimethylhydrazine (DMH) at a dose of 20 mg/kg body weight for 30 weeks. The result showed that in DMH and fenugreek treated group the activity of β -glucuronidase was decreased compared to the control which had no fenugreek supplementation. β-glucuronidase is the intestinal bacterial enzyme which can hydrolyse toxins and mutagens, releasing the active carcinogens into the entero-hepatic circulation. This inhibition of β-glucuronidase activity by fenugreek seeds could be due to its fiber content or due to the anticarcinogenic components like flavonoids and saponins (Fujiki *et al.*, 1986; Jain and Aggarwal, 1990).

RATIONALE

Diabetes is a multi-factorial metabolic disease, characterized primarily by glucose metabolism abnormalities that lead to the development of acute and long term complications. The incidences of acute complications (diabetic ketoacidosis, lactic acidosis) are currently decreasing due to pharmacological therapies (Stamler *et al.*, 1993). However, long term complications of macroangiopathy (ischaemic heart disease, peripheral arteropathy stroke) and microangiopathy (nephropathy, retinopahty) are rapidly increasing, thus accounting for the higher mortality for cardiovascular events in diabetic patients compared to the general population (DeFronzo and Ferrannini, 1991; Morrish *et al.*, 2001). Poor blood glucose control has been shown to be an important risk factor for the development of vascular diabetic complications.

Temporary hyperglycemia after a meal can have prolonged effects on the development of atherosclerosis (Gavin, 2001). The postprandial phase is characterized by the occurrence of oxidative stress due to the increased plasma glucose levels; this condition increases plasma insulin, triglyceride and oxidized LDL levels (Ceriello, 2000). In diabetic individuals, a light or moderate postprandial hyperglycemia produces more intensive oxidative stress than in non-diabetic individuals, because in the former, all the metabolic responses are amplified with a cumulative deleterious effect on the vascular endothelium. Endothelium dysfunction has been found followed by changes in the vascular endothelial matrix, cell differentiation and proliferation, endothelium permeability and expression of adhesion molecules. The increase in molecule adhesion and the accumulation of advanced glycosylation end products (AGE) has been suggested to promote the migration of macrophages into the sclerotic lesions which is responsible

for the inflammatory reaction associated with plaque development (Ceriello *et al.*, 1999; Ceriello, 2000).

Although many pharmacological agents are available to lower blood glucose, the normalization of blood glucose for any appreciable period of time is seldom achieved (Turner *et al.*, 1999). In addition, in well-controlled so-called "intensively" treated patients, serious complications may still occur (Reichard, *et al.*, 1993; UK, 1998), and the economic and personal burden of diabetes remains. Furthermore, microvascular disease is already present in many individuals with undiagnosed or newly diagnosed T2D (Harris, 1993).

Consideration of the role of diet on intestinal adaptation in diabetes mellitus (DM) is important because of the possibility of altering glucose and lipid absorption to reduce hyperglycemia and hypercholesterolemia (Thomson and Wild, 1997). It has been reported that glucose absorption is increased in poorly controlled diabetic subjects (Vinnik *et al.*, 1965; Damci *et al.*, 2003). Similarly, the intestinal uptake of glucose and lipids was reported to be enhanced in diabetic rats (Thomson and Wild, 1997). Small intestine motility is also reported to be abnormal in diabetic subjects and this potentially affects glucose absorption (Rayner *et al.*, 2002). The enhanced uptake was returned to normal by treatments that reduced glycemia and/or improve intestinal motility, such as treating with exogenous insulin (Kellett *et al.*, 1984) or feeding diets containing soluble fiber (Johnson and Gee, 1981), or polyunsaturated fatty acids (Thomson *et al.*, 1988; Churnratanakul *et al.*,1991).

A clinical goal of treating individuals with diabetes is to decrease postprandial hyperglycemia and cardiovascular risk factors (i.e. hyperlipidemia, hypertension, visceral

adiposity and insulin-resistance). Diet plays a major role in preventing the rapid rise and falls of plasma glucose levels in the postprandial state. Specifically, modifications in the type and amount of dietary fiber have been demonstrated to help with both glycemic control and dyslipidemia (Olson *et al.*, 1997; Brown *et al.*, 1999; Anderson *et al.*, 2000).

The concept of self-treatment with alternative medicines and therapies has generated a renewed interest in plants compounds. Some familiar food products possess compounds with medicinal properties. This has stimulated research and development interest on these plants in both pharmaceutical and food manufacturing industries. One of the potential natural sources for exerting anti-diabetic and anti-hyperlipidemia activity is the fenugreek seed.

In recent years, fenugreek has been introduced to Canada as a crop. Cultivation of this plant on a small scale started in Western Canada at the Lethbridge Research Center and has shown that it can be a cash crop for farmers (Basu *et al.*, 2003). Despite the versatility of fenugreek as a crop, the cultivation acreage in Canada remains small (500 hectors in Western Canada) (Slinkard, 2002; Basu *et al.*, 2003). This could partly be due to the fact that the health benefits of Canadian fenugreek have not been determined. The known glucose and lipid-lowering effects of the Asian grown seed suggest that the Canadian grown fenugreek may also be useful as an adjunct in the treatment of T2D and hyperlipidemia. As a novel crop in Canada, fenugreek has considerable economic and medical potential particularly in the nutraceuticals and food industries.

Although health benefits of fenugreek have been documented, its seeds are not currently recommended by the medical profession. This may be due to the lack of large studies to make evidence-based recommendations, and the undesirable physical

properties of the seeds. For example, fenugreek seeds are bitter and have a pungent maple odor. The bitter taste and pungent odor of fenugreek are mainly due to the oil, steroidal saponins, and alkaloids present in the seed (Madar, 1987). There is also little known about the effective dose. Many studies were performed using whole or defatted fenugreek seeds at doses ranging from 5-100 g/day in either normal, T2D, or hypercholesterolemic subjects (Mardar *et al.*, 1988; Sharma *et al.*, 1990). This dose would not be possible to achieve in a dietary supplement. Before further human trials can be conducted it is necessary to overcome the physical problems of providing the active ingredient in fenugreek seeds. This thesis will determine a method to extract galactomannan (a major bioactive component) from fenugreek seeds and then investigate the hypoglycemic and hypocholesterolemic effects of this extract using an animal model.

OBJECTIVES AND HYPOTHESES

The overall objective of this thesis was to investigate the possibility of using galactomannan extracted from Alberta-grown fenugreek seeds for food application and biological effect. The first part, Objective 1, was aimed to extract galactomannan from Alberta-grown fenugreek seeds. The extracted product was assessed for its viscosity and viscoelastic properties. The second part, Objective 2, was focused on determining the biological effect of the galactomannan extract on inhibiting rate of glucose uptake (*in vitro*), and on glycemic and lipid status (*in vivo*)

Objective 1:

This objective was accomplished in three studies. The first study focuses on the comparison of chemical compositions among four lines of Alberta-grown fenugreek seeds and Indian fenugreek seeds. Specifically, galactomannan content was compared. One of four lines of Alberta-grown fenugreek seeds was chosen for galactomannan extraction study. The second study was to develop extraction methods to obtain high yield and purity of galactomannan. The third study was to determine viscosity and viscoelastic properties of galactomannan extract.

It was specifically hypothesized that:

I. The chemical compositions of Alberta-grown fenugreek seeds were not different from Indian grown fenugreek seeds.

II The fractionated-extraction method provided a greater yield and purity of galactomannan than the chemical-extraction method.

III Viscosity and viscoelastic properties of galactomannan would not be affected by adding 10% and 20% (w/w) sucrose.

Objective 2:

The galactomannan extract obtained from objective 1 was used to determine for the biological effect in rats. This objective included two studies: *in vitro* and *in vivo* studies. The *in vitro* study was aimed at determining the dose relationship of galactomannan to inhibit glucose uptake in intestine of JCR rats. The *in vivo* study was to investigate the effect of feeding galactomannan on glycemic and lipid status of Sprague-Dawley rats.

It is specially hypothesized that:

IV Galactomannan extract would inhibit glucose uptake *in vitro* in the intestine of both lean and obese JCR rats.

V Feeding galactomannan solution together with glucose solution would reduce plasma glucose level of rats.

VI Feeding a diet with galactomannan would reduce plasma glucose, insulin, cholesterol, TG, and adipose weight of rats.

Chapter Format

The hypotheses posed were tested in a sequence of experiments. These experiments were organized as thesis chapters. Chapter 2 has been submitted for scientific publication in the International Journal of Food Sciences and Nutrition.

Objective 1:

Chapter 2 (test of hypotheses I and II)

The chemical compositions of fenugreek seeds were determined using 4 lines of Alberta-grown fenugreek seeds and compared to that of Indian fenugreek seed which was used as a reference (*hypothesis* I). All seeds were ground to obtain powder that passed through a 0.5 mm mesh sieve. It was hypothesized that the chemical compositions of fat, protein, soluble fiber, insoluble fiber, ash, amino acid profiles, fatty acid profiles, galactomannan and saponins were not different among all fenugreek lines (*hypothesis* II). This chapter described the development of two methods for the extraction and purification of galactomannan from a line of Alberta-grown fenugreek seed.

Chapter 3 (test of hypothesis III)

The galactomannan extract obtained from chapter 2 was examined for viscosity and viscoelastic properties. Different concentrations of galactomannan solution (0.25%, 0.5%, 0.75%, and 1%, w/w) were tested at different temperatures (4°C, 10°C, 25°C, 37°C, and 50°C). Sucrose at 10% and 20% (w/w) concentrations were added to all concentrations of galactomannan solutions and tested under different temperatures for its viscosity and viscoelastic properties.

Objective 2

Chapter 4 (test of hypothesis IV)

Galactomannan extract at different concentration (0.1%, 0.25%, 0.35%, and 0.5%, w/w) was used to determine its inhibitory effect on the rate of glucose uptake *in vitro* in intestine of JCR rats. Both jejunum and ileum from lean and obese JCR rats were used. Each galactomannan solution was mixed with either 2 mM or 32 mM glucose in buffer solution and used for incubation of jejunum and ileum tissues.

Chapter 5 (test of hypotheses V and VI)

The effect of feeding galactomannan-containing glucose solution on plasma glucose levels of rats was determined using healthy Sprague-Dawley rats (*hypothesis* V). The rats were gavaged with 75% (w/w) glucose solution in a dose of 3 g/kgBW. In the following weeks, rats were gavaged with 75% (w/w) glucose solution (3 g/kgBW) added with different concentrations of galactomannan extract (0.5% and 1%, w/w). All rats had a week of wash-out period between each test. This chapter described the effects of feeding diets with or without galactomannan on plasma concentrations of glucose and lipid of Sprague-Dawley rats, using control, control diet with either 2.5% or 5% (w/w) galactomannan (*hypothesis* VI). The control diet was a high sucrose diet (52%, w/w). Galactomannan containing diets were made by replacing cellulose with either 2.5% or 5% (w/w) galactomannan. After 3 weeks of feeding, the rats were given an oral glucose tolerance test (OGTT) to determine the effect on plasma glucose and insulin levels. The rats were continually fed for 4 weeks.

Chapter 6 summarizes the findings of these hypotheses and provides general discussion.

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Chapter 2

Composition analysis of fenugreek seeds and development of galactomannan extraction methods

2.1 Introduction

Fenugreek (*Trigonella foenum-graecum* L.) is an annual dicotyledonous plant belonging to the family Leguminosae, sub-family Papilionaceae. Fenugreek pods contain 10 to 12 brown or yellowish seeds. Until recently, it has been cultivated mainly in India, Pakistan, France, Argentina, and Northern Africa. Fenugreek seeds contain sapogenins, 4-hydroxyisoleucine and galactomannans, which have been shown to modify hyperglycemia and hypercholesterolemia in both animals and humans (review in Srinivasan, 2006).

The potential health benefits of fenugreek have led many researchers to explore the growth and market potential of this plant (Srinivasan, 2006). Fenugreek was recently adapted to be cultivated in Southern Alberta, Canada. The Lethbridge Research Center of Agriculture-Canada has been successful in cultivating a number of lines of fenugreek. Nutrient compositions of these lines have never been investigated nor have their potential health benefits for humans.

The first objective of this study was to determine the nutrient compositions among the four fenugreek lines (Amber, F-70, F-86, and L-3314) grown in Southern Alberta. The second objective was to develop a convenient method for the extraction and purification of galactomannan from seeds of a selected fenugreek line. Seeds of fenugreek grown in India have been used in the majority of health-related investigations

reported in literature (Sharma *et al.*, 1996; Srinivasan, 2006) and hence the seeds of an Indian line were used in this study as reference.

2.2 Materials and methods

2.2.1 Materials

Fenugreek seeds of four experimental lines, including, Amber, F-70, F-86, and L-3314, were obtained from Agriculture and Agric-Food Canada, Lethbridge Research Center (Lethbridge), Alberta. Seeds of an Indian line were obtained from a local grocery store. All seeds were ground to pass through a 0.5 mm mesh sieve and the ground seeds were stored in airtight containers in a freezer at -20°C until they were used. Galactomannan kits were purchased from Megazyme Co., Ireland. All chemicals used in this study were of analytical grade and procured from Sigma Chemical Co., St. Louise, MO.

2.2.2 Proximate analysis

Lipids of a ground seed sample (35 g) was subjected to extraction with hexane in a Soxhlet apparatus for 12 hours at 70°C. Solvent was evaporated in a rotary evaporator (Buchi, Model RE 121, Switzerland), weight of the lipid residue was recorded and the crude lipid content was expressed as % w/w.

Total nitrogen content of a 1 g ground seed sample was determined using Technicon Industrial nitrogen determination procedure 146/71A and the standard conversion factor of 6.25 for vegetable protein (Sosulski and Imafidon, 1990) was used to calculate the crude protein content.

Sample (2 g) in a pre-weighed crucible was charred on a hotplate and then ignited in a muffle furnace set at a temperature of 550°C for 14 hours. Weight of the ash content was expressed as % w/w.

Two grams of ground seeds in a pre-weighed aluminum dish was dried in an oven at 105°C for 2 hours. The moisture content was determined on the basis of weight difference (before and after drying) and expressed as % w/w.

2.2.3 Determination of soluble and insoluble dietary fiber

An assay kit designed to analyze dietary fiber was used for this determination (AOAC, 1992). A 1 g sample was mixed with 50 ml of phosphate buffer (pH 6) and 0.1 ml of liquid α-amylase in a conical flask. The mixture was incubated for 15 min at 95°C. The pH of the mixture was adjusted to 7.5 ± 0.2 with a solution of 0.275 N sodium hydroxide. A 0.1 ml of a solution of protease (50mg/ml) in phosphate buffer were added and incubated at 60°C for 30 min. The pH of the mixture was adjusted to 4.0-4.6 range with a solution of 0.325 M hydrochloric acid. Amyloglucosidase (0.1 ml) was added to the mixture and incubated at 60°C for further 30 min. The mixture was filtered through crucible containing 0.1 mg of Celite® and the residue containing insoluble fiber was dried overnight in an oven at 100°C. Crude protein and mineral content of the dried residue was determined and subtracted of from the residue weight to calculate insoluble fiber content. For soluble fiber determination, ethanol (95% v/v, 4 x the volume of filtrate) was added to the filtrate and left standing overnight at room temperature (RT) to allow complete precipitation. Contents were then passed through a Celite filter and the residue washed with three 20 ml portions of 78% (v/v) ethanol, two 10 ml portions of

95% (v/v) ethanol, and two 10 ml portions of acetone. Protein and mineral contents of the residue were determined and subtracted from the weight of residue to obtain the soluble fiber content.

2.2.4 Determination of fatty acid profile

Fatty acid profile of the lipids was determined in a gas chromatography (GC). One milli-liter of methylating reagent (methanol, acetyl chloride and benzene; 20:1:4, v/v) was added to each tube for extraction and methylation of the oil (Kaushik and Agnihotri, 1997). The methyl esters were dissolved in 50 µl hexane. A 1 µl sample was injected into GC, which equipped with a SP 2560 capillary column (100 m x 0.25 mm id, 0.2 µ film thickness; Supelco, Bellefonte, PS) according to the method of Yurawecz *et al.* (1999) with modifications. Oven temperature was initially set at 220°C for 10.25 min and then ramped to 240°C at 30°C/min and held for 9 min. Injector and flame ionization detector temperatures were set at 270°C. Carrier gas was helium at a flow rate of 15 ml/min.

2.2.5 Determination of amino acid profile

Proteins in a 20 mg ground seed sample were first hydrolyzed into free amino acids with 6N hydrochloric acid according to the method of Llames and Fontaine (1994). Amino acid profile was then determined in an amino acid analyzer (Waters 717 plus autosampler, Waters Corporation, Millford, MA). A Waters 3.9 mm x 150 mm AccQ.Tag Amino Acid Analysis column-containing 4 micrometer silica base particles with C18 hydrocarbon was used at a temperature of 37°C. Mobile phase A (1 liter)

contained 19.0g sodium acetate trihydrate, 6.9 ml of concentrated phosphoric acid, 1.72 ml of concentrated triethylamine, 0.1g sodium azide and 72.28 ml of high performance liquid chromatography (HPLC) grade water. Mobile phases B and C were acetonitrile and HPLC grade water, respectively. The gradient used was 100% A for 1 min, 1.0% (v/v)A, 95.1% (v/v) B and 4.9% (v/v) C for 19 min, 1.0% (v/v) A, 91% (v/v) B and 9% (v/v)C for 5 min, 1.0% (v/v) A, 83.0%(v/v) B and 17%(v/v) C for 10 min, 60%(v/v) B and 40%(v/v) C for 9 min, 100% A for 3 min, and 60% (v/v) B and 40%(v/v) C for 9 min. Total run time was 55 min and the internal standard was L-α-amino-n-butyric acid. The injection volume was 10 µl (Anonymous, 1995).

2.2.6 Determination of sapogenins

Defatted and dried seed powder was transferred into a test tube. Sapogenins were extracted with 5 ml of 80% (v/v) ethanol and hydrolyzed for 2 hours with 2 mL of 1M sulfuric acid in 70% (v/v) propanol. Water (3 mL) and 50 µg of 6-methyldiosgenin internal standard were added, extracted with methyl *tert*-butyl ether (3 x 1 ml), solvent evaporated at 30°C in a Meyer N-EVAP apparatus (Organomation Associates, Berlin, MA), weight of the residue recorded and the residue was dissolved in 1 ml of toluene. A portion (2 µl) of this dissolved solution was analyzed in a GC (Hewlett-Packard, HP 6890) equipped with an HP-5 column (30 m x 0.32 mm i.d.), an HP 6890 series autoinjector, a flame ionization detector (FID, 300°C), and an electronic gas control. The split/splitless injection port, operated at 250°C, was equipped with a silanized glass liner (HP part 5181-3316). The sample was injected directly (30 seconds) at an initial oven temperature of 200°C and then ramped to 290°C at a rate of 1°C min^{-1}. The carrier gas

was helium with 2 ml min^{-1} constant flow under electronic pressure control (Taylor *et al.*, 2000). Retention times and peak area counts were obtained with HP GC ChemStation software (version A.05.04). Confirmation of peak identity was obtained by mass spectrometry, using HP 5989A GC-MS as reported previously by Taylor *et al.* (1997). Sapogenin content was expressed as % (w/w) and its composition was expressed as % relative GC peak area.

2.2.7 Determination of galactomannans

Content of galactomannans was determined using the method supplied for the galactomannan kit, Megazyme Co. (McCleary, 1981). Samples (80 mg) were treated with 80% (v/v) aqueous ethanol and incubated at 85-90°C for 5 min. These treated samples were centrifuged at 1,000 x g for 10 min. The pellets were treated with 8 ml of 50 mM sodium acetate buffer (pH 4.5), placed in a boiling water bath for 5 min and then incubated at 40°C for 1 hour. The samples were treated with 15 µl of authentic β-mannanase (300 U/ml in ammonium sulphate) and incubated at 40°C for 1 hour. After centrifugation, three 0.1 ml aliquots of the supernatant were transferred into three test tubes and 0.2 ml of acetate buffer (pH 4.5, 50 mM) was added into each tube. Twenty micro-liters of purified guar seed α-galactosidase (150 units/ml) were added into two tubes and the same volume of acetate buffer was added into the third tube (blank) before incubating at 40°C for 1 hour. The sample was then treated with 2.5 ml of 200 mM Tris buffer (pH 8.6), 0.1 ml of aqueous nicotinamide adenine dinucleotide (0.1 g/10 ml), and 7 µL of galactose dehydrogenase (70 units/ml, Roche Diagnostics, Basel, Switzerland) and then incubated at 40°C for 1 hour. The absorbance at 340 nm was measured against a

blank solution using a diode array spectrophotometer (Model 8452A, Hewlett Packard, CA).

2.2.8 Galactose and mannose (G:M) ratio

G:M ratio was calculated using GC peak areas for galactose and mannose in hydrolyzed galactomannan samples according to the method of Lien *et al.* (1996) with modifications. Samples (50 mg) were incubated in 3 ml sulfuric acid (12 mol/L) for 30 min at RT. The samples were diluted to 3 mol/L with the addition of water, purged with nitrogen, and hydrolyzed at 110°C for 1 hour. After hydrolysis, 200 µl of *myo*-inositol (Sigma) at 10 g/L was added as internal standard. Aliquots (1 ml) of the hydrolysate were made basic with the addition of 0.7 ml concentrated NH_4OH. Sugars were reduced by adding 100 µl of the basic samples to 1 ml sodium borohydride (30 g/L in dimethylsulfoxide; Sigma) and heating at 40°C for 90 min. After the decomposition of excess sodium borohydride with glacial acetic acid (200 µl), 1-methylimidazole (0.2 ml) and then acetic anhydride (2 ml) were added and acetylation allowed proceeding for 10 min. Acetylation was stopped with the addition of 5 ml water and the mixture cooled to room temperature. Alditol acetates were extracted into 4 ml dichloromethane, rinsed twice with water (5 ml), and evaporated to dryness under a stream of nitrogen. Samples were redissolved in 1 ml dichloromethane before analysis on a DB-17 capillary column 0.25 mm internal diameter x 30 m (J&W Scientific, Folsom, CA) in a Varian 3400 gas-liquid chromatograph.

2.2.9 Fractionation of fenugreek seeds

The primary and secondary processing steps involved in the preparation of galactomannan samples from whole seeds and fractions (obtained by milling and sieving of whole seeds) is presented in Figure 2-1.

Fenugreek seeds were separated into two fractions namely fraction A (seed coat/aleurone/endosperm) and fraction B (cotyledon + axis) using a Quaker City Mill (Model 4-E, The Straub Co., PA). Each fraction was ground to pass through a 0.5 mm mesh sieve and subjected to nutrient analyses as described previously.

2.2.10 Extraction and purification of fenugreek gum from whole seeds

Fenugreek powder (35 g, 0.5 mm mesh) was subjected to extraction with n-hexane in Soxhlet apparatus for 12 hours (Fig 2-1). The residue was air-dried for 4 days at RT. Defatted fenugreek powder was treated with reverse osmosis (RO) applying solid to water ratio of 1:40 w/v, and then placed in a boiling water bath for 15 min to inactivate β-D-mannanase, which causes a rapid fall in viscosity (Ganter *et al.,* 1997). The slurry was removed and cooled to RT. The pH of this slurry was adjusted to 11 by adding 1N sodium hydroxide (NaOH), agitated at RT for 4 hours and followed by centrifugation at 17,700 x g for 30 min. The supernatant was transferred into a beaker, pH adjusted to 4.58 with concentrated hydrochloric acid (HCl), stirred at RT for 15 min, and centrifuged at 17,700 x g for 30 min. Supernatant was transferred into a beaker, pH adjusted to 7 with 1N NaOH and stirred for 15 min at RT. The final supernatant was treated with absolute ethanol in a ratio of 1:2 v/v, and left standing overnight at 4°C. The resulting precipitate was removed, 200 ml of absolute ethanol were added and homogenized using

a polytron to obtain a precipitate with a fine particle size. The homogenized precipitate was then left standing for 1 hour and filtrated under vacuum. The residue containing fenugreek gum was subjected to nutrient analyses as explained previously.

2.2.11 Extraction and purification of fenugreek gum from seed fractions A and B

Fractions A and B (0.5 mm mesh size) were dispersed separately in RO water at a ratio of 1:40 (w/v) and placed in a boiling water bath for 15 min to inactivate β-D-mannanase (Fig 2-1). Fractions were then cooled to RT, agitated for 4 hours and centrifuged at 17,700 x g for 30 min. The supernatant was transferred into a beaker, treated with absolute ethanol at a ratio of 1:1 (v/v) and held overnight at 4°C. The precipitate was removed, 200 ml of absolute ethanol added to the supernatant, which was then homogenized using a polytron to obtain a precipitate with a fine particle size. After standing for 1 hour, the mixture was vacuum filtered to recover the fenugreek gum.

The following formulas were used to calculate yield of gum and, recovery and purity of galactomannan of whole seeds and fractions:

Yield = (WG/WSM)*100

Recovery = (WGG/WGSM)*100

Purity = (WGG/WG)*100

where, WG, WSM, WGG, and WGSM denote weight of gum, weight of starting material, weight of galactomannans in gum and weight of galactomannans in starting material, respectively.

2.2.12 Statistical analysis

In the present study, fenugreek seeds of four lines were obtained separately and harvested in the same year in Lethbridge. The lot number of the Indian line was shown on the package, but there was no information in regard to harvesting.

Four samples were analyzed from each line. The chemical compositions of raw and fractionated seeds were presented as means ± SEM (sample size, n=4). Proximate compositions of galactomannan extract were analyzed using analysis of variance (ANOVA) and the differences among mean values were established using Student-Newman-Keuls (SNK) test and considered significant at $p < 0.05$.

2.3 Results and discussion

2.3.1 Composition of fenugreek seeds

The nutrient and fiber compositions of five lines (including a line of Indian origin) of fenugreek seeds are shown in Table 2-1. Lipid content of these lines ranged from 7% to 9.6% (w/w); these values were similar to the data reported by Brummer *et al.* (2003), Mansour and El-Adawy (1994), and Sharma (1986). These researchers reported lipid content of 7%, 7%, and 8% (w/w) respectively. Crude protein content in seeds of four lines grown in Southern Alberta was 29-32% (w/w) while the Indian line was 26% (w/w). Seeds used in this study had a high protein content as compared to fenugreek seeds from Egypt (24% w/w, Ullmann, 1987), and India (26% w/w, Sharma, 1986), but they were slightly lower than that (34% w/w) reported by Brummer *et al.* (2003). Soluble, insoluble and total dietary fiber contents of the fenugreek seeds used in the present study, were found to contain 16%-22% (w/w), 26%-32% (w/w) and 42-48%

(w/w), respectively. These data were comparable to those of Rao *et al.* (1996) who reported soluble, insoluble and total fiber of fenugreek seeds at 20%, 28%, and 48% (w/w) respectively.

2.3.2 Fatty acid profiles

Major fatty acid entities of these five lines were oleic acid (39-48% relative peak area) and linoleic acid (30-38% relative peak area) (Table 2-2). Each of these fatty acids represented 3-4% (w/w) and 3% (w/w) of whole seed, respectively. Indian line had significantly low content of linoleic acid ($p<0.05$) among five lines. Oleic acid and linoleic acid contents in seeds used in this study were slightly higher than that reported in the review of Ravindran (1997) who reported 35% of oleic and 38% linoleic acid. Ravindran (1997) also reported a linolenic acid content of 14%, a slightly higher content when compared to seeds used in this study. Baccou *et al.* (1978) investigated the fatty acid profile of fenugreek seeds from different countries. They reported that the fatty acid profiles are dependent upon the geographical location and conditions of cultivation.

2.3.3 Amino acid profiles

Food and Agriculture Organization and World Health Organization (FAO/WHO, 1973) reported that 36% of total protein being total essential amino acid (TEAA) in any seed makes that seed a good source of protein. TEAA contents in seeds of Alberta-grown lines were higher than that of the Indian line and were higher than the FAO recommendation (Table 2-3). Content of methionine, a limiting amino acid in legumes, in whole seeds was higher (0.7-1.1%) than 0.6% reported by Mansour and El-Adawy

(1994). The observed differences in crude protein and amino acid contents were possibly due to the use of different lines grown in different geographical locations and the employment of different analytical techniques. Deosthale *et al.* (1970) reported that an excess leucine in foods interferes with the utilization of isoleucine and lysine. However, leucine:isoleucine (1.4:1) of the fenugreek seeds used in this study was lower than the FAO/WHO (1973) recommended ratio of 1.8:1.

2.3.4 Content of sapogenins

Seeds of Amber and Indian lines had content of sapogenins in the range of 0.3-0.5% (w/w) of whole seed (Table 2-4). Diosgenin was the predominant steroidal sapogenins in all five lines. Seeds used in this study contained neotigogenin/β-sitosterol mixture at higher levels (9-13% w/w of total sapogenins) compared to 6% and 7% (w/w of total sapogenins) reported by Taylor *et al.* (1997) and Brenac and Sauvaire (1996), respectively. Smilagenin, sarsasapogenins, yuccagenin, gitogenin were minor constituents of sapogenins in the lines investigated. These observations were similar to those of Petit *et al.* (1995) who detected smilagenin and sarsasapogenin as minor components in fenugreek seeds.

2.3.5 Content of galactomannans

The galactomannan content of the four Alberta-grown lines ranged from 33-37% (w/w, Table 2-5). All four lines grown in Southern Alberta had higher amount of galactomannans than 22%, 20%, and 19% (w/w) reported by Brummer *et al.* (2003), Sharma (1986), and Valette (1984), respectively. G:M of four lines grown in Southern

Alberta ranged from 1:1.3 to 1:1.4 while Indian line had higher galactose substitutions in galactomannan structure (1:1.1). However, G:M in the seeds used in this study is in agreement with that reported by Cui (2001). Mardar and Shomer (1990) also reported G:M of endosperm fraction to be 1:1.5 while that of the seed coat fraction to be 1:1.1.

2.3.6 Galactomannan extraction methods

Using the Amber line of Alberta fenugreek seeds, the present study was further extended to develop methods for the extraction and purification of galactomannan. The Amber line was chosen because its seeds are available in large quantities.

Galactomannan was extracted using a conventional method, that is, using chemical reagents. Our method was initiated by discussing with researchers (Drs Temelli, Nakano, Li, Burkus and Mr.Moquin) experienced in β-glucan extraction in the Department of Agricultural, Food and Nutritional Science, University of Alberta, Canada. Fenugreek seeds were first investigated for the optimal time of eliminating fat content by using hexane extraction in Soxhlet apparatus. The result showed that the optimal time for elimination fat in fenugreek seeds is 12 hours (Appendix 1). We tried to develop and test procedures for obtaining pure galactomannan extract. Some of them are shown in appendix 2-4. We used Papain® (Sigma Chemical Co., St. Loise, MO) to eliminate protein, but the final product had high protein contamination (6-11%, w/w). Na_2CO_3 was then used to eliminate protein by aiming to reach isoelectric point (pI) of protein, but this reagent makes foam during pH adjustment. Therefore, NaOH was replaced to overcome foaming problem.

Our preliminary investigation with three different extraction temperatures of 4°C, 23°C, and 100°C indicated that temperature had no significant effect on the fenugreek gum yield. This observation differed somewhat from the observations by Brummer *et al.* (2003) who reported a gum yield of 22% (w/w) at a temperature of 10°C and extraction time of 2 hours, and by Ramesh *et al.* (2001) who reported a gum yield of 19% (w/w) at a temperature of 100°C and extraction time of 4 hours. These discrepancies may be attributed to differences in G:M ratios. A high degree of galactose substitution results in better dispersion and solubilization of gum in water at RT. The Amber line used in this study had G:M of 1:1.4 indicating a high degree of substitution. The gum extracted from defatted fenugreek seeds at RT (23°C) with an extraction time of 4 hours had a yield of 16% (w/w) on seed weight basis, comparable to that reported by Ramesh *et al.* (2001). The different yields could also be attributed to varietal differences.

Alkali extraction method used in this study was a modification of the technique of Bardalaye and Nordin (1977). These authors have reported that alkali condition facilitates dissolution of isolated galactomannans from *Aspergillus niger*. At a high pH, hydroxyl groups become ionized, converting neutral polysaccharides to polyelectrolytes. This, in turn, has an expanding effect on molecular dimensions through intramolecular electrostatic repulsion (Goycoolea *et al.*, 1995). In this study, 1N NaOH, used for the adjustment of pH to 11 served two purposes. One was the facilitation of galactomannan dissolution while preventing the protein dissolution. Our preliminary study revealed that most of the proteins in fenugreek seeds were water-soluble. Our results also showed that protein-contaminated fenugreek gum had brown color and rigid nature. During the extraction, pH was adjusted to 4.53 in order to eliminate the majority of proteins with

isoelectric points (pI) in the range of 4.25-4.75. After centrifugation, the last supernatant was adjusted to neutral pH and precipitated with absolute ethanol to obtain fenugreek gum with a high purity galactomannan. The purification process was sufficient to afford a white color gum with a purity of 93% (w/w) and a yield of 16% (w/w on seed basis). Crude protein content was reduced from 32% (w/w) for whole seeds to 3% (w/w), whereas lipid content was only 0.3% (w/w) as compared to 7% (w/w) in the seed origin. There were no substantial changes in mineral content (Tables 2-1 and 2-7). Since the crude gum obtained in this method had a white color with light and puffy appearance, it is suitable for both nutraceutical/functional food products.

Although galactomannan can be successfully extracted by using a conventional method, it was time consuming and inefficient for scale-up production. The product obtained from this method was sensitive to adjusted pH. It was also difficult to control to obtain standardized outcome if pH adjustments are varied. We studied the structure of fenugreek seed to determine the location of galactomannan in the seed (Appendix 5).

In the second extraction method (fractionated method), seeds of fenugreek were separated into two fractions namely fraction A (seed coat/aleurone/endosperm) and fraction B (cotyledon + axis) using a Quaker City Mill as described in section 2.2.9. The yield of fraction A and B was 30.5% and 69.5% (w/w, whole seed weight basis), respectively. Table 2-6 shows that fraction A had a low content of crude lipids, crude proteins and minerals compared to fraction B while galactomannan were located predominantly in the fraction A. The galactomannan in fraction B were originated from the residual endosperm surrounding the axis.

Our preliminary study involving with or without 1N sodium hydroxide to adjust pH to 11 during the dissolution of both fractions showed that there were no significant differences in yields afforded. Dea and Morrison (1975) reported that strong alkali facilitates dissolution of otherwise water-insoluble unsubstituted mannan in ivory (*Phytelephas macrocarpa*) nuts. Similarly, Ramesh *et al.* (2001) used 1N NaOH to extract gum from the residue of fenugreek seed after treating it with water at RT and 100°C. They reported a gum yield of 11% (w/w) with 12% (w/w) contaminants.

Table 2-7 shows the composition of fenugreek gum extracted from both fractions A and B. Data suggested that the purification process was better in eliminating impurities of fraction A than in fraction B. Fenugreek gum extracted from fraction A contained 91.4% (w/w) galactomannan which had 3% (w/w) protein contamination. This is in agreement with the data of Brummer *et al.* (2003) who reported 2.4% (w/w) of protein contaminants in the fenugreek gum. The yield of fenugreek gum from fraction A was 48% (w/w) with the final product being white in color whereas fraction B had 10% (w/w) yield with the final product being brown in color. The brown color of gum obtained from fraction B was partly due to a high degree of protein contaminants (52% w/w). The yield of fenugreek gum extracted from fraction A was higher than 22% (w/w) reported by Brummer *et al.* (2003) and, 11% w/w (cold water extraction) and 19% w/w (hot water extraction) reported by Ramesh *et al.* (2001). These differences may be attributed to the geographical location of cultivation, growing conditions (Reid, 1985), quantity in starting material and method of extraction (Reid, 1970). Galactomannan that was extracted from fraction A using the fractionated extraction method provided similar quality product as those extracted from a conventional method. The fractionated extraction method, unlike

the conventional method, does not need chemical reagents, therefore it is environment friendly and cost-effective. The cost is CAD$ 0.43/g of galactomannan extract. This is beneficial for scaled-up production of galactomannan.

In summary, data for fraction A and B of the Amber line reveal that galactomannans are predominantly located in fraction A (seed coat/aleurone/ endosperm). Varieties of fenugreek included in this study are high in galactomannans (33-37% w/w) and crude proteins (29-32% w/w) compared to the Indian grown seed and the levels presented in the literature. Fenugreek seed contains linoleic acid (3% w/w) and oleic acid (3-4% w/w) as predominant fatty acids. Among the three different extraction methods investigated, extraction involving fraction A afforded the highest recovery of galactomannans (89% w/w, on the basis of total galactomannan) while extractions involving whole seed and fraction B afforded relatively low recoveries (46% and 47% w/w, respectively). These indicated that galactomannans are predominantly located in fraction A, which consisted of seed coat, aleurone and endosperm. The use of fraction A to extract galactomannans was efficient for both yield and cost effectiveness.

Table 2-1. Proximate composition of fenugreek seeds (%, w/w, dry basis)

Component	Fenugreek Lines				
	Amber	F-70	F-86	L-3314	Indian
Crude lipids	7.0 ± 0.3	8.1 ± 0.9	8.3 ± 0.5	8.4 ± 0.5	9.6 ± 0.8
Crude proteins	31.6 ± 0.8	28.7 ± 0.3	30.1 ± 0.5	31.6 ± 0.2	26.0 ± 0.3
Minerals	3.4 ± 0.1	3.5 ± 0.4	3.2 ± 0.3	3.5 ± 0.3	3.2 ± 0.2
Soluble fiber	18.8 ± 0.2	21.7 ± 0.3	16.1 ± 0.3	18.2 ± 0.3	17.5 ± 0.8
Insoluble fiber	25.8 ± 0.3	25.8 ± 0.4	32.3 ± 0.5	27.4 ± 0.6	28.1 ± 0.1

Data present as mean ±SEM, (n=4)

Table 2-2. Fatty acid profile of fenugreek seed (% Relative GC peak area, dry basis)

Fatty acid	Fenugreek Lines				
	Amber (% relative)	F-70 (% relative)	F-86 (% relative)	L-3314 (% relative)	Indian (% relative)
Palmitic ($C_{16:0}$)	9.4 ± 0.3	11.0 ± 0.3	8.5 ± 0.3	10.1 ± 0.3	9.9 ± 0.4
Stearic ($C_{18:0}$)	1.9 ± 0.3	2.6 ± 0.2	1.9 ± 0.3	1.8 ± 0.3	2.1 ± 0.3
Oleic ($C_{18:1}$)	40.0 ± 0.3	39.0 ± 0.3	41.2 ± 0.3	39.5 ± 0.3	47.6 ± 0.4
Linoleic ($C_{18:2}$)	38.0 ±1.7	35.6 ± 0.5	37.9 ± 3.1	36.6 ± 0.4	29.8 ± 0.3
Linolenic ($C_{18:3}$)	10.4 ± 1.0	11.2 ± 0.1	11.0 ± 0.2	11.8 ± 0.3	10.5 ± 0.9
Arachidonic ($C_{20:0}$)	ND	0.6 ± 0.2	ND	ND	ND

Data present as mean ± SEM, (n=4)
ND = value was too small to be detected

Table 2-3. Amino acid profile of fenugreek seed proteins (mg/g protein, dry basis)

Amino acid	Fenugreek Lines				
	Amber	F-70	F-86	L-3314	India
Lysine	63.0 ±0.6	78.0 ± 4.3	69.6 ± 5.5	58.1 ± 2.8	64.5 ± 0.8
Isoleucine	48.7 ± 0.8	61.3 ± 0.6	51.4 ± 4.3	50.2 ± 7.6	51.8 ± 0.8
Leucine	67.7 ± 0.8	86.1 ± 6.5	71.6 ± 2.4	70.1 ± 1.8	73.3 ± 0.8
Cysteine	16.1 ± 1.4	17.1 ± 1.4	16.6 ± 0.9	16.4 ± 2.0	17.3 ± 0.8
Methionine	10.4 ± 2.2	11.1 ± 0.3	10.6 ± 0.4	10.7 ± 0.4	10.4 ±1.0
Tyrosine	23.4 ± 1.7	28.9 ± 1.5	23.9 ± 0.5	23.7 ± 1.0	25.3 ± 0.4
Phenylalanine	41.1 ± 0.4	50.9 ± 1.3	42.4 ± 0.8	43.9 ±0.5	45.7 ± 0.5
Valine	39.5 ± 0.1	49.1 ± 0.3	41.5 ± 0.7	40.1 ±0.2	43.4 ± 0.5
Threonine	36.7 ± 0.2	44.2 ± 0.3	37.1 ± 0.6	37.0 ± 0.8	40.7 ± 1.0
Tryptophan	13.3 ± 0.1	10.1 ± 0.6	10.9 ± 0.6	14.8 ± 0.8	12.3 ± 0.2
Serine	49.7 ± 0.5	61.3 ± 0.2	50.7 ± 0.7	50.5 ± 0.3	55.3 ± 0.3
Glutamic acid	167.1 ± 0.2	207.0 ± 1.4	176.8 ±1.1	162.7 ±1.2	169.7 ±0.6
Glycine	46.5 ±0.5	56.1 ± 1.0	47.4 ± 0.3	47.7 ± 0.6	55.3 ± 0.2
Histidine	25.0 ± 0.4	30.7 ± 0.7	26.5 ± 0.2	25.9± 0.9	28.8 ± 0.9
Arginine	102.5 ± 0.9	125.4 ± 1.1	107.8 ± 1.2	107.7 ±1.0	101.0 ± 1.4
Aspartic acid	106.3 ± 0.5	132.4 ± 1.8	114.8 ± 1.3	103.3 ±1.2	110.2 ±0.9
Alanine	38.3 ± 0.6	47.0 ± 1.5	39.1 ± 0.3	36.3 ± 0.6	39.9 ± 1.2
Proline	44.9 ± 0.9	56.1 ± 0.3	46.8 ± 1.1	46.4 ± 0.7	47.2 ± 1.3
TEAA	35.9	43.5	37.2	36.5	26.1
L:I	1.4:1	1.4:1	1.4:1	1.4:1	1.4:1

Data present as mean ± SEM, (n=4)
TEAA= Total essential amino acids
L:I = Leucine:isoleucine ratio

Table 2-4. Sapogenin content and its composition of fenugreek seeds

Component	Fenugreek Lines				
	Amber	F-70	F-86	L-3314	Indian
(%, w/w, dry seed basis) Sapogenins	0.4 ± 1x10^{-2}	0.3 ±1x10^{-2}	0.3 ± 1x10^{-2}	0.3 ± 1x10^{-2}	0.5± 2x10^{-2}
(% relative GC peak area)					
-Smilagenin	2.7± 0.5	4.7± 0.3	2.6± 0.8	5.9± 0.5	1.7± 0.1
-Sarsa sapogenin	1.6± 0.3	3.1± 1.1	1.8± 0.7	3.7± 0.2	1.2± 0.1
-Diosgenin	47.8±1.6	41.0±5.1	43.9± 2.9	44.6± 2.1	43.8± 3.2
-Tigogenin	6.1± 0.3	6.3± 0.5	7.7± 0.7	7.2± 0.8	9.0± 1.5
-Yamogenin	23.5 ±1.5	21.6 ± 1.1	21.3± 1.2	18.8± 0.7	20.6± 2.1
-Neotigogenin β-sitosterol mixture	9.5±0.2	12.5± 1.4	12.3±0.7	12.6±1.5	9.7± 0.4
-Yuccagenin	2.3±0.4	2.2± 0.8	1.7±0.7	2.2± 0.6	2.7± 0.6
-Gitogenin	5.1 ± 0.9	6.5 ± 1.8	7.0± 1.2	4.5± 1.2	9.1± 2.5
-Neogitogenin	1.3± 0.3	2.1± 0.9	1.8± 0.5	0.5± 0.9	2.2± 0.7

Data present as mean ± SEM, (n=4)

Table 2-5. Galactomannan content (% w/w, dry basis) and G:M among fenugreek lines

Line	Galactomannan	G:M
Amber	32.8 ± 3.1	1:1.4
F-70	37.1 ± 2.2	1:1.4
F-86	33.0 ±2.0	1:1.3
L-3314	36.8 ±2.2	1:1.4
India	23.1 ±0.7	1:1.1

Data present as mean ± SEM, (n=4)

Table 2-6. Proximate composition of fractionated fenugreek seeds (% w/w, dry basis)

Component	Fraction A (seed coat/aleurone/endosperm)	Fraction B (cotyledon/axis)
Galactomannans	48.5 ±2.8	7.9 ± 0.1
Crude proteins	3.8 ± 0.4	31.5 ± 1.2
Crude lipids	0.4 ± 0.1	8.7 ± 0.8
Ash	2.2 ± 0.1	3.4 ± 0.2

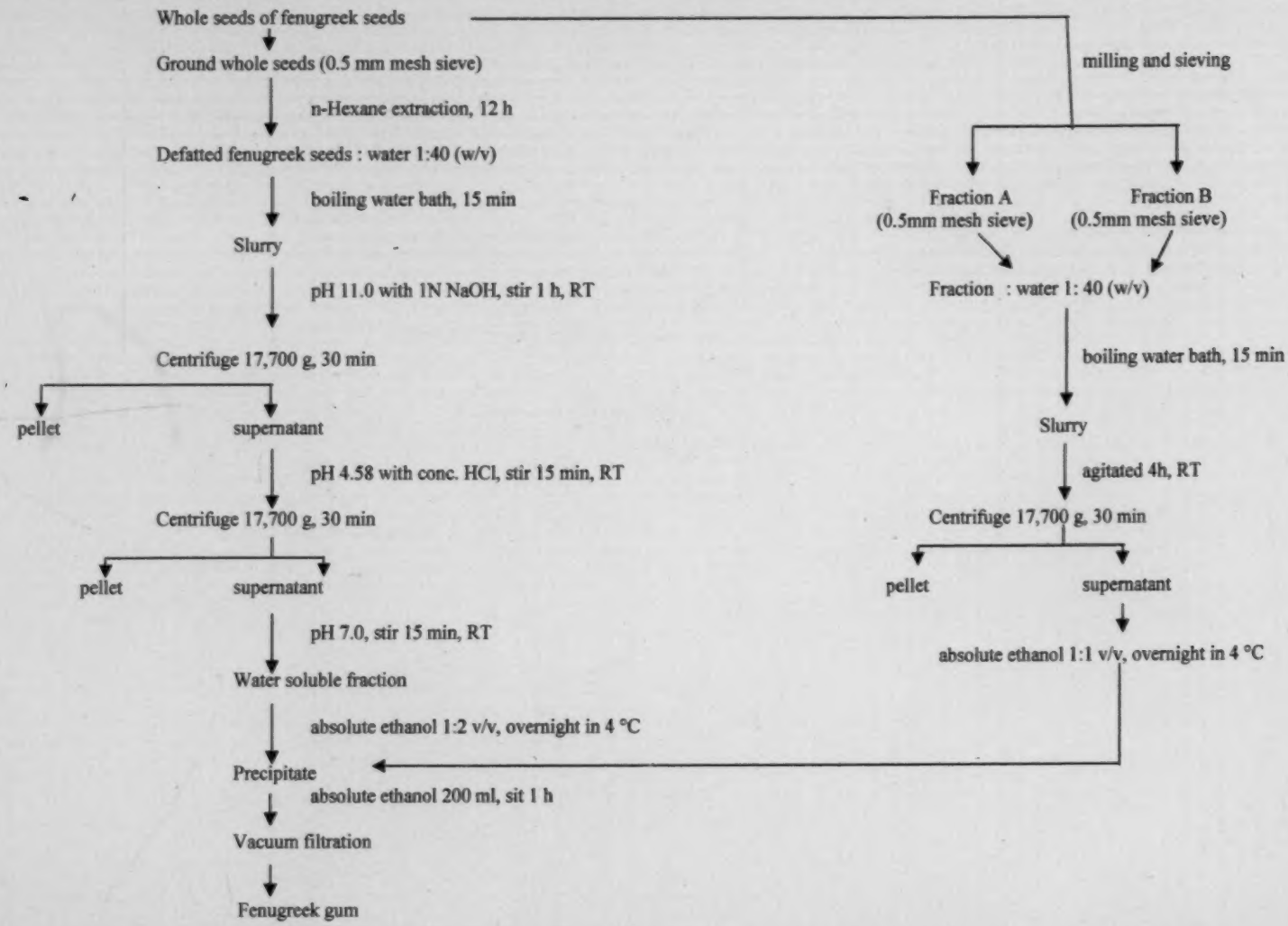
Data present as mean ± SEM, (n=4)

Table 2-7. Proximate composition of fenugreek gum extracted among whole seeds, fraction A and fraction B (% w/w, dry basis)

Component	Whole seeds	Fraction A (seed coat/aleurone/endosperm)	Fraction B (Cotyledon + axis)
Galactomannan	93.0 ± 2.2^{a}	91.4 ± 2.9^{a}	35.4 ± 2.7^{b}
Crude proteins	3.3 ± 0.2^{b}	2.8 ± 0.1^{b}	51.8 ± 3.4^{a}
Crude lipids	0.3 ± 0.2	ND	0.2 ± 0.0
Minerals	3.1 ± 0.1^{b}	0.5 ± 0.06^{c}	7.7 ± 0.1^{a}

[a-b] Means sharing the same superscript in a row are not significantly different ($p>0.05$) from one another. Each value was averaged from 6 samples, which were produced in 3 different days.
ND= value was too small to be detected

Figure2-1 Processing steps and extraction procedure involved in the preparation of galactomannan samples from whole seeds, fraction A and B.



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Chapter 3

Rheological properties of galactomannan gum

3.1 Introduction

Galactomannans are the most abundant and commercially important seed gums; they are used as thickening and gelling agents in food products (Closs *et al.*, 1999; Cunningham and Walsh, 2002). The very high viscosities attained at low concentrations and its greater ability to associate in solutions make galactomannans use almost exclusively as an additive in foods and pet foods (Kok *et al.*, 1999).

The gelation mechanism of gums is strongly influenced by co-solutes such as sugars (Duran *et al.*, 1994; Papageorgiou and Kasapis, 1995) or salts (Grasdalen and Smidsrod, 1987; Milas *et al.*, 1990). Studies showed changes in the rheological and thermal properties of gums when sugar or salt was added in the solution (Richardson and Norton, 1998; Doyle *et al.*, 2006). Sucrose can increase the dynamic Young's modulus and the melting point of kappa-carrageenan gels (Nishinari and Watase, 1992), but reduces the gel strength of a blend of kappa-carrageenan and locust bean gums (Fiszman and Duran, 1992). Sucrose in concentrated aqueous solutions tends to pack in an orderly fashion that may have a stabilizing effect on the orderly packed hydrocolloid suspensions (Papageorgiou *et al.*, 1994). Watase *et al.* (1990) reported an increase in dynamic elastic modulus of agarose gels with increasing sugar concentrations up to a certain point and a decrease thereafter with excessive additions of sugars. Sucrose can be readily crystallized in aqueous solutions under proper conditions. In the crystal structure, the

hydroxyl groups of sucrose, except for O-4, form intramolecular and intermolecular hydrogen bonds (Brown and Levy, 1963).

Fenugreek-galactomannan obtained from our previous study (chapter 2) has not been investigated for its viscosity and viscoelastic properties. The general objective of the research was to determine the inhibiting effect of galactomannan on sugar absorption in the GIT. Therefore, the viscosity and viscoelastic properties of galactomannan were determined both in the presence and absence of sucrose in the solution. High concentrations of sucrose (10% and 20%, w/w) were chosen because it was previously shown that 5-10% (w/w) sucrose mixed with guar-galactomannan had no effect on the viscosity (Sudhakar *et al.*, 1996).

The objective of the present study was to examine the viscosity and gel formation of fenugreek-galactomannan gum dispersions in the presence and in the absence of sucrose at varying temperatures.

3.2 Materials and methods

3.2.1 Materials

Fenugreek-galactomannan gum of 91.4% (w/w) purity was fractionated in our laboratory (chapter 2). Sucrose was obtained from Sigma Chemical Co.(St. Louise, MO).

3.2.2 Sample preparation

Aqueous (RO water) galactomannan solutions at concentrations of 0.25%, 0.5%, 0.75% and 1% (w/w) were prepared with gentle stirring in a boiling water bath for 30 min to ensure homogeneity.

3.2.3 Determination of viscosity and thixotropy

Viscosity measurements were recorded at consecutive fixed shear rates of 1.29-129 s^{-1} using a Parr Physica UDS 200 rheometer (Glenn, VA). The viscometer was equipped with a Peltier heating system that controlled the sample temperature. All viscosity measurements were recorded at 4°C, 10°C, 25°C, 37°C, and 50°C using DG 27 cup and bob geometry (n =4). The shear rate was reported in s^{-1} after multiplying the rpm value by a conversion factor of 1.29 s^{-1} provided by the manufacturer.

Thixotropy of gum dispersions at 4°C, 10°C, 25°C, 37°C, and 50°C was determined using DG 27 cup and bob geometry (n=4). These determinations were performed using a series of fixed shear rates that consecutively increased from 1.29 to 3870 s^{-1} and then immediately decreased to the original shear rate of 1.29 s^{-1}. All analyses were performed in duplicates.

3.2.4 Determination of viscoelastic properties

Fenugreek-galactomannan gum dispersions were prepared using the method explained in 3.2.2. Gum dispersions were allowed to equilibrate for 15 min at RT. The storage and loss moduli (n=4) were recorded at 4°C, 10°C, 25°C, 37°C, and 50°C using a 7±0.005 g sample placed in a DG 27 cup and bob geometry of a Parr Physica UDS 200 rheometer set in amplitude sweep controlled shear displacement mode with a constant frequency of 10 Hz and controlled strain of 0.1-100%.

3.3 Results and discussion

Fenugreek galactomannans are hydrophilic molecules. Theoretically, the substitution rate of G:M (1:1.4) in fenugreek galactomannan will make it to have high-water binding capacity, forming very viscous solutions at relatively low concentration when compared to other galactomannans (Evan, 1992). Galactomannan in the present study shows shear-thinning behavior and non-newtonian flow, particularly in pseudoplastic sub-class. This means that the viscosity of the solution decreases with increasing rate of shear (Bourne, 2002). Pseudoplastic flow appears to be important in contributing to good sensory qualities (i.e., mouthfeel and flavor release to foods). It is also important in the pourability of suspension and emulsion. Based on information of guar gum which has similar structure of galactomannan to fenugreek galactomannan, the very high viscosities attained at low concentrations make guar gum an excellent thickener in the food industry, such as in soups, desserts, pie fillings (Dierckx and Dewettinck, 2002). However, the viscosity of guar gum may be markedly reduced by the presence of sucrose (Elfak *et al.*, 1979). Since sugars, salts, and other strongly hydrophilic components can hydrate rapidly and compete for water molecules, inhibiting hydration and formation of a molecular dispersion of gums (Whistler and BeMiller, 1997). The results in this study showed that the viscosity of fenugreek galactomannan was not reduced when it was mixed with 10% and 20% (w/w) sucrose solutions. This suggests that galactomannan extracted from fenugreek seeds has potential use in the food industries.

3.3.1 Viscosity, flow behaviour index and consistency coefficient of galactomannan gum dispersions

Table 3-1 shows the viscosity data of galactomannan gum dispersions in the presence and absence of added sucrose. At a concentration of 0.25% (w/w), galactomannan gum dispersions showed low viscosity of 8-35 mPa·s, depending on the shear rate and the measuring temperature. When the concentration was increased from 0.25% (w/w) to 1% (w/w) at 0.25% (w/w) increments, the viscosity of the gum dispersions increased non-linearly. At 1% (w/w) concentration, gum dispersions exerted viscosities of 172-1590 mPa·s, depending on the shear rate and the measuring temperature. Added sucrose did not affect the viscosity of galactomannan gum dispersions of up to 0.75% (w/w) concentration while it negatively affected the viscosity of 1% (w/w) dispersions. This implies that galactomannan concentrations up to 0.75% (w/w) can be used in high sucrose products, such as jams and jellies. However, the addition of sucrose to obtain galactomannan gum dispersions with higher viscosity is not necessary.

To acquire the flow behaviour properties of galactomannan gum dispersions, the Power law model ($S = cR^n$, where, S is the shear stress in Nm^{-2}, R is the shear rate in s^{-1}, c is the consistency coefficient and n is the flow behaviour index or Power law index) was employed (Table 3-2). Gum dispersions having flow behaviour index >1 exert Newtonian flow behaviour, whereas those having flow behaviour index <1 are pseudoplastic liquids (Marcotte *et al.*, 2001).

Galactomannan dispersions exerted pseudoplastic liquid properties as the flow behaviour index for all dispersions at all measurement temperatures were <0.93 with the

lowest being 0.31 for 1% (w/w) dispersions at 4°C and no added sucrose (Table 3-2). Pseudoplasticity became more prominent with increasing concentrations of galactomannan in the dispersions. In general, adding sugar had very little or no effect on the pseudoplastic property of dispersions and this is a positive outcome in terms of its food applications. As expected, pseudoplastic property of dispersions decreased with increasing measurement temperature and the effect was more pronounced at galactomannan concentrations >0.5% (w/w).

Consistency coefficient followed an opposite trend as compared to the flow behaviour index (Table 3-3). It increased substantially when galactomannan concentration increased from 0.25% (w/w) to 1% (w/w) at increments of 0.25% (w/w). This parameter was heavily dependent upon the measurement temperature as it decreased substantially at higher temperatures. Added sugar also negatively affected the consistency coefficient with the effect being most pronounced when 20% (w/w) were added.

3.3.2 Thixotropy of galactomannan gum dispersions

Thixotropy is an important rheological property associated with the isothermal sol↔gel transformation (Nishinari and Watase, 1992). It is defined as the decrease in viscosity due to the collapse of three-dimensional network upon applying a constant shear rate or consecutive fixed shear rates over a period of time followed by the regaining of the network structure, and hence the viscosity when shear is withdrawn (Muller, 1973; Schramn, 1994). Non-thixotropic gum dispersions are characterized by their ability to maintain constant viscosity at fixed shear rates. Their viscosity decreases upon

subjecting to consecutively increasing shear rates, but they can regain the viscosity over time. As depicted in Figure 3-1, galactomannan gum dispersions were non-thixotropic because they regained the initial viscosity (at 1.29 s^{-1}) when the shear rate was decreased from 3870 s^{-1} to 1.29 s^{-1}. This indicates that galactomannan can be used in the foods, which require high shear during the manufacturing process.

3.3.3 Elastic and loss moduli of galactomannan gum dispersions

Elastic modulus (G′) and loss modulus (G″) were associated with the viscoelastic properties of gum dispersions. G′ and G″ at controlled strain and constant frequency (10 Hz) were recorded in order to locate the linear viscoelastic region of the gum dispersions (Mandala and Palogou, 2003). A constant frequency of 10 Hz was employed to allow adequate time for the polymer network to form and break because the polymer chains cannot disentangle during the short time periods of oscillation at high frequencies (Lazaridou *et al.*, 2003). The linearity can be disturbed when the gum suspension is strained to a point where certain weak bonds of the network structure are destroyed; similarly the formation of new bonds can also affect the linear viscoelastic region. It is known that the non-cross linked gum dispersions have a shorter linear region than cross-linked gums (Dickinson and Merino, 2002).

The data obtained for galactomannan concentrations up to 0.5% (w/w) did not show distinct G′ and G″ patterns and these were erratic. This could be attributed to the application of relatively high frequency for low concentration dispersions. However, distinct G′ and G″ patterns were observed for 0.75% and 1% (w/w) dispersions. For 0.75% (w/w) concentration at 4°C and 10°C, G′ was greater than G″ indicating the

formation of an elastic gel-like network at low temperatures (Figure 3-2). At 25°C, 37°C, and 50°C, G″ was greater than G′ showing the viscoelastic liquid-like properties at relatively high temperatures (Figure 3-2). At 1% (w/w) concentration, the dispersions behaved like elastic gel-like networks regardless of the measuring temperature because G′ was greater than G″ (Figure 3-2). Formation of elastic gel-like network is important for food applications while the formation of viscoelastic liquid at physiological temperature can contribute to the increased viscosity in digesta.

In summary, viscosity of fenugreek-galactomannan gum dispersions is not affected by added sucrose (up to 20% w/w) and a gum concentration (up to 0.75% w/w), indicating that the addition of sucrose is not necessary to achieve high viscosity. This also implies that fenugreek-galactomannan gum can be used in products that require high sugar concentrations. In addition, the flow behavior index and the consistency coefficient of fenugreek-galactomannan dispersions were not affected by the added sucrose and all dispersions at varying concentrations confer pseudoplastic liquid properties. These properties coupled with non-thixotropic nature make the fenugreek gum suitable for a wide range of food and non-food applications. Furthermore, the ability of fenugreek-galactomannan gum dispersions to behave like viscoelastic liquid and elastic gel-like networks at different concentrations and at various temperatures (RT and physiological temperature) makes them ideal for functional foods, targeting the blood glucose regulation through highly viscous digesta.

Table 3-1. Viscosity of pure galactomannans gum as affected by the solute concentration, shear rate and measuring temperature

Shear rate (1/s)	Temperature (°C)	Viscosity (mPa·s)			
		0.25% gum (w/w)	0.5% gum (w/w)	0.75% gum (w/w)	1% gum (w/w)
0% sugar					
12.9	4	35	231	686	1590
	10	27	205	638	1500
	25	19	152	528	1310
	37	14	121	449	1170
	50	10	95	385	1020
129	4	19	70	149	288
	10	17	65	142	275
	25	12	54	123	252
	37	10	46	114	236
	50	8	40	102	218
10% sugar					
12.9	4	32	225	691	1400
	10	26	199	641	1320
	25	17	143	522	1140
	37	13	110	441	1010
	50	10	92	368	888
129	4	19	72	152	261
	10	17	65	142	248
	25	12	53	124	222
	37	10	44	113	205
	50	8	38	100	191
20% sugar					
12.9	4	34	182	629	1240
	10	29	159	575	1170
	25	19	123	452	995
	37	14	96	375	890
	50	10	74	303	774
129	4	21	65	146	244
	10	19	57	135	227
	25	13	48	113	201
	37	10	40	100	186
	50	8	33	89	172

Data present as mean, each value was averaged from 4 samples
mPa·s = milli pascal second

Table 3-2. Flow behaviour index of aqueous dispersions of pure galactomannans gum as affected by solute concentration and temperature

Gum concentration (%, w/w)	Temperature (°C)	Flow behaviour index (n)		
		0% sugar (w/w)	10% sugar (w/w)	20% sugar (w/w)
0.25	4	0.828	0.810	0.852
	10	0.815	0.876	0.865
	25	0.867	0.883	0.892
	37	0.863	0.912	0.908
	50	0.853	0.912	0.933
0.5	4	0.592	0.592	0.637
	10	0.605	0.594	0.641
	25	0.654	0.647	0.675
	37	0.678	0.685	0.709
	50	0.724	0.714	0.736
0.75	4	0.397	0.406	0.423
	10	0.406	0.419	0.437
	25	0.458	0.465	0.484
	37	0.495	0.504	0.523
	50	0.508	0.531	0.566
1	4	0.308	0.312	0.324
	10	0.323	0.326	0.333
	25	0.362	0.361	0.368
	37	0.392	0.393	0.393
	50	0.428	0.423	0.430

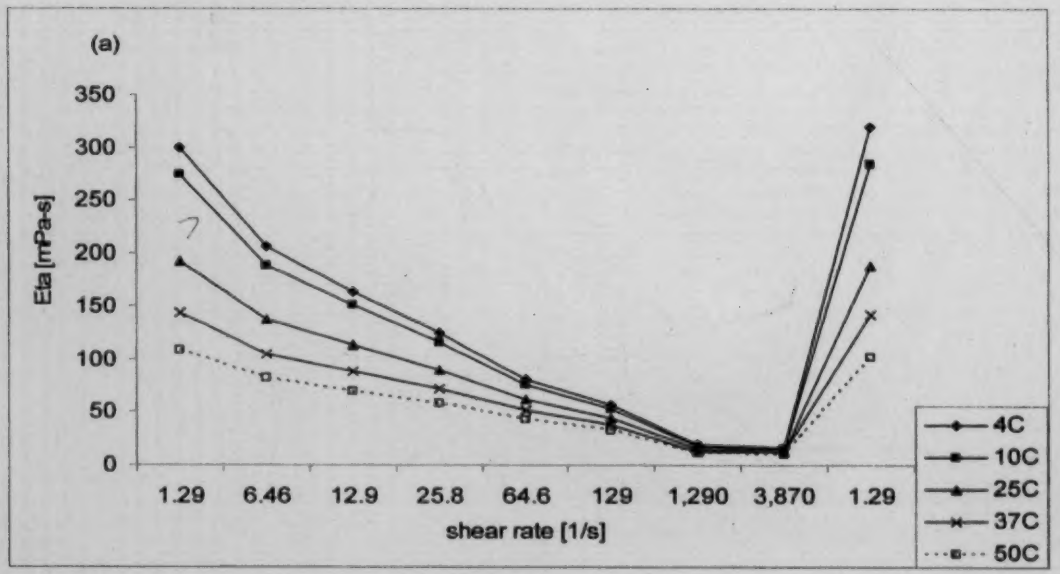
Data present as mean, each value was averaged from 4 samples

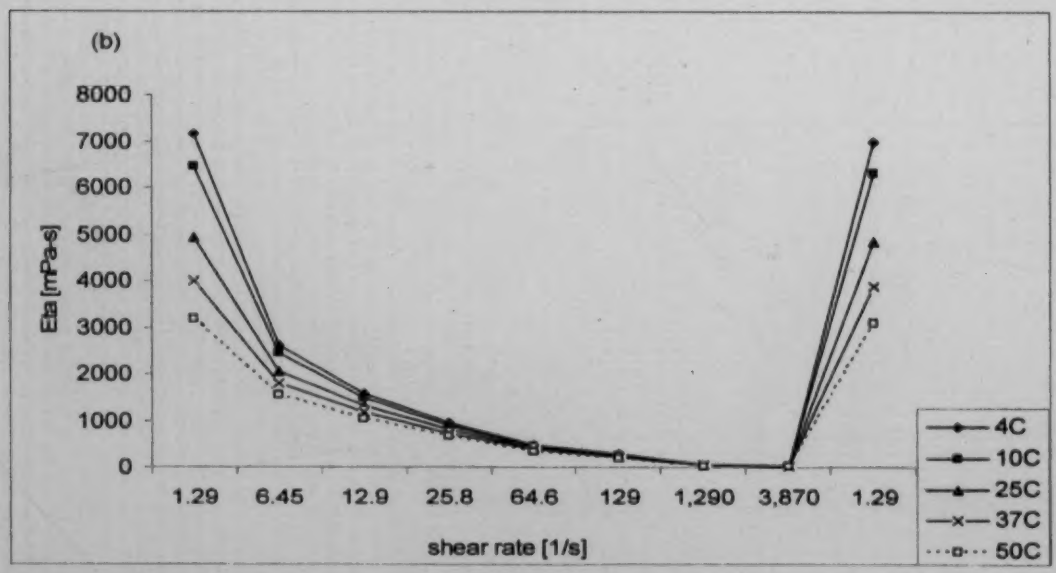
Table 3-3. Consistency coefficient of aqueous dispersions of pure galactomannans as affected by solute concentration and temperature

Galactomnnans (%, w/w)	Temperature (°C)	Consistency coefficient (c)		
		0% sugar (w/w)	10% sugar (w/w)	20% sugar (w/w)
0.25	4	0.047	0.050	0.047
	10	0.043	0.033	0.039
	25	0.024	0.022	0.024
	37	0.019	0.015	0.017
	50	0.016	0.012	0.012
0.5	4	0.560	0.576	0.417
	10	0.484	0.512	0.363
	25	0.310	0.322	0.256
	37	0.233	0.225	0.184
	50	0.166	0.175	0.134
0.75	4	3.012	2.917	2.565
	10	2.739	2.603	2.243
	25	1.929	1.852	1.534
	37	1.494	1.407	1.143
	50	1.276	1.098	0.823
1	4	8.804	7.727	6.710
	10	7.916	6.947	6.095
	25	6.118	5.395	4.647
	37	5.023	4.326	3.850
	50	3.952	3.504	3.018

Data present as mean, each value was averaged from 4 samples

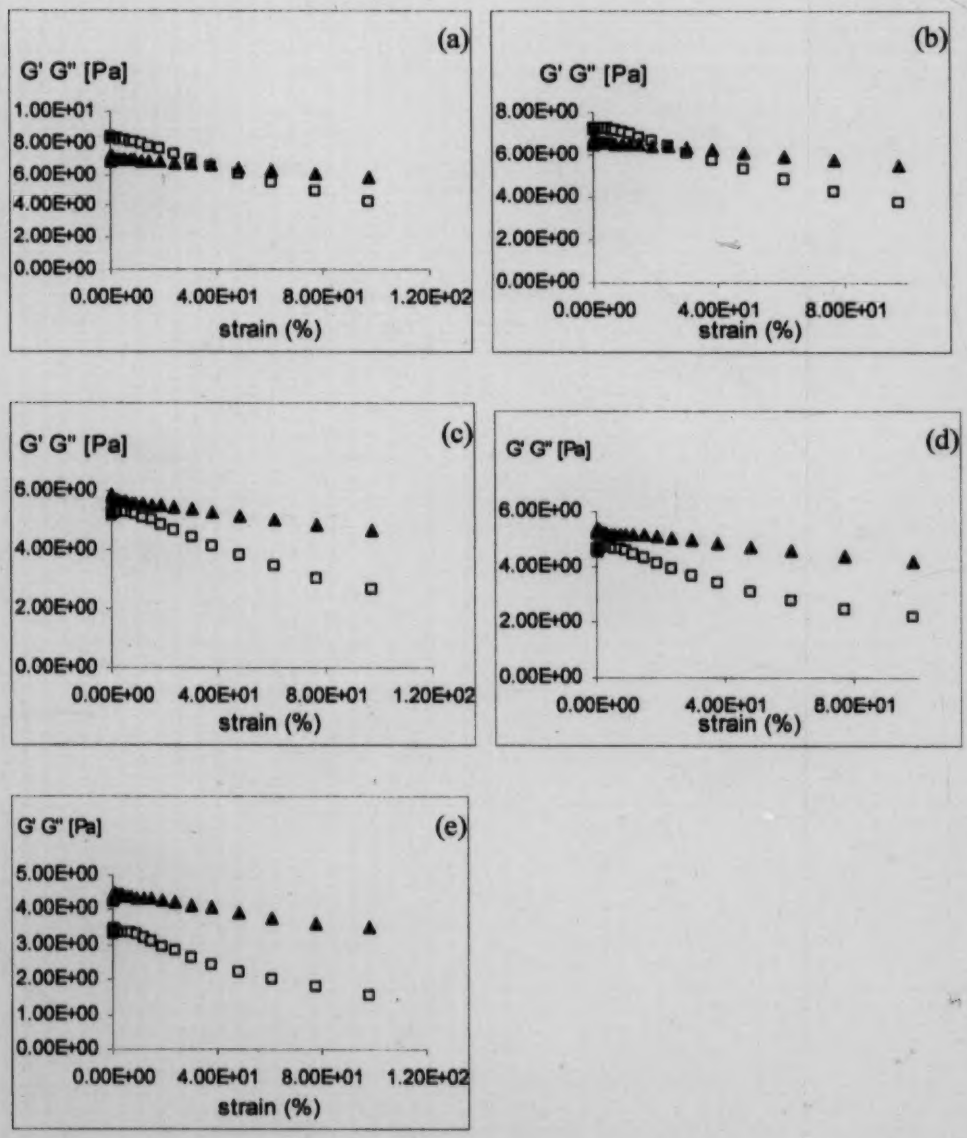
Figure 3-1 Non-thixotropic performance of (a) 0.5% (w/w) galactomannan gum (b) 1.0% (w/w) galactomannan gum as affected by shear rate and measuring temperatures





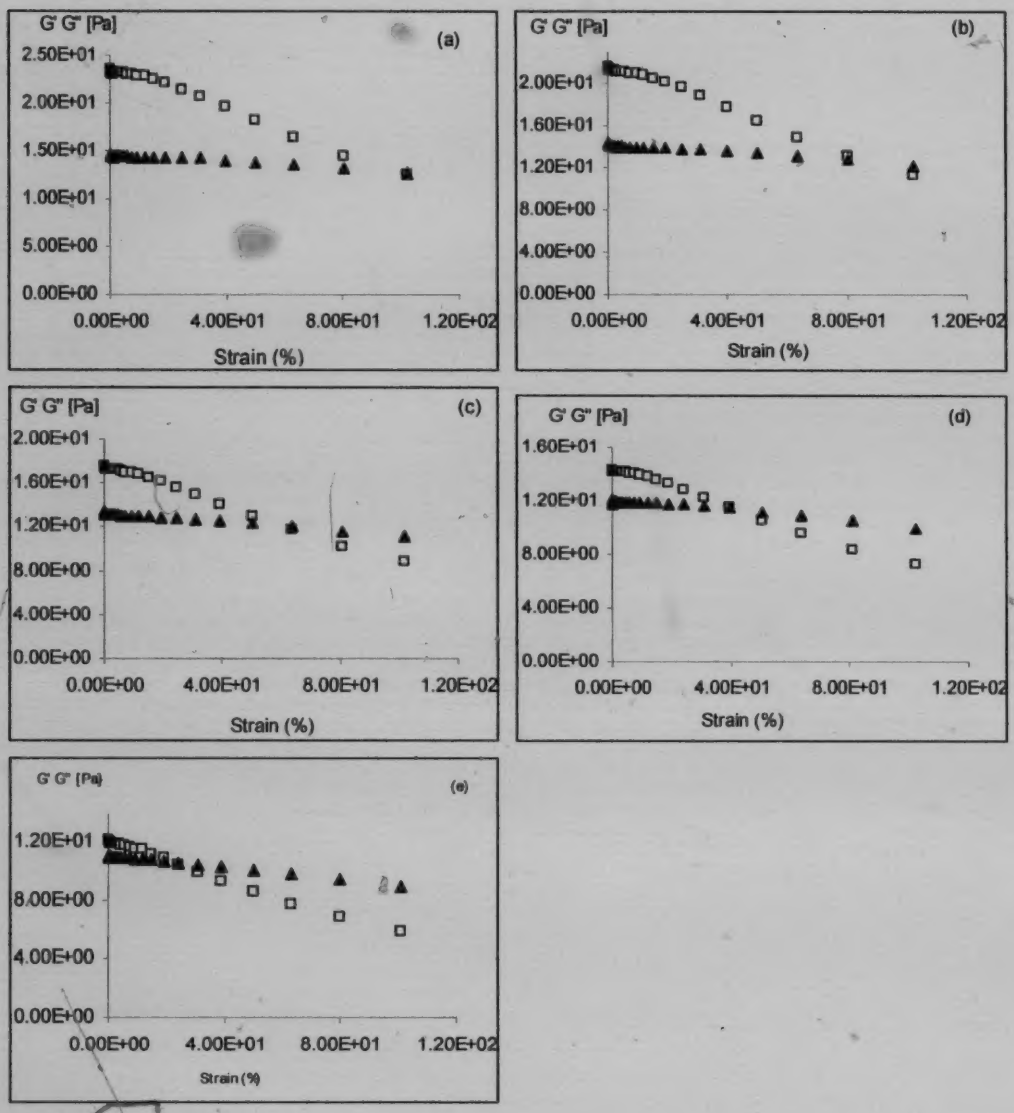
Data present as mean, each value was averaged from 4 samples
Eta = apparent viscosity
mPa·s = milli pascal second

Figure 3-2 Elastic modulus(G',□)and loss modulus (G",▲) performed of 0.75% (w/w) galactomannan at different temperatures (a) 4°C (b) 10°C (c) 25°C (d) 37°C (e) 50°C



Data present as mean, each value was averaged from 4 samples
Pa= pascal

Figure 3-3. Elastic modulus(G',□)and loss modulus (G", ▲) performed of 1.0% (w/w) galactomannan at different temperatures (a) 4°C (b) 10°C (c) 25°C (d) 37°C (e)50°C



Data present as mean, each value was averaged from 4 samples
Pa = pascal

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Chapter 4

An *in vitro* effect of galactomannan on the intestinal glucose uptake in rats

4.1 Introduction

Galactomannan (i.e. guar gum) has been shown to reduce postprandial blood glucose in both animals (Cameron-Smith *et al.*, 1994) and humans (Jenkins *et al.*, 1978; Jarjis *et al.*, 1984). The proposed mechanism suggests that the presence of soluble fibers in the small intestine increase the viscosity of the digesta, and the increase appears to be proportional to the concentration of the fiber (Johnson and Gee, 1981; Blackburn *et al.*, 1984; Eastwood and Morris, 1992). Viscous fibers may also delay gastric emptying (Jenkins *et al*, 1978; Fuse *et al*, 1989), and delay nutrient absorption in the small intestine (Blackburn *et al*, 1984; Rainbird *et al.*, 1984; Flourie *et al.*, 1984). The mechanism of action remains controversial and may be influenced by several external factors. Some *in vitro* and *in vivo* studies have shown that viscous fibers did not reduce the rate of transport of solute across intestinal epithelium (Forster and Hoos, 1977; Elsenhans *et al.*, 1980; Schwartz and Levine, 1980). Leclere *et al.* (1994) indicated that the viscosity of guar gum did not reduce the accessibility of luminal contents toward the absorptive mucosal surface. One of the reasons for these controversies may lie in the study design such as the type and concentration of galactomannan used, which in turn gives different viscosities in the lumen.

The JCR-LA cp/cp rat is a model of obesity, insulin resistance, cardiovascular plaque formation, and atherosclerosis (Russell, 2001). The nomenclature of corpulent JCR rat [JCR: LA-*Lepr*^{cp}] were originally developed by crossing the obese spontaneously

hypertensive (SHROB) rat model with the normotensive strain derived from Koltesky's original (LA/N) rat model in the laboratory of Dr. Carl Hansen at the National Institutes of Health (NIH). The partially backcrossed model at generation F5 was sent to Dr. James C. Russell at the University of Alberta in 1978 and to Charles River Inc. in 2003. These rats have been widely used for many metabolic studies in relations to insulin resistance and cardiovascular disease (Russell, 2001; Deng *et al.*, 2004).

Results in chapter 3 demonstrated that the viscosity of galactomannan increased when its concentration was increased. However, this increase was not directly proportional to the increase in concentration. Various concentrations of galactomannan may therefore have different effects on inhibiting glucose absorption. Using the purified galactomannan extract derived from fenugreek seeds (chapter 2), an *in vitro* study was designed to examine the rat intestinal uptake of glucose. The first objective of this study was to determine the dose relationship of galactomannan on the inhibition of glucose uptake in both the jejunum and ileum. Three factors including the dose of galactomannan (0, 0.1%, 0.25%, 0.35%, and 0.5%, w/w), the concentration of glucose in the solution (2 and 32 mM), and the genotype of rats (lean and obese JCR rats), were investigated in a 5 x 2 x 2 factorial design with two replications. The second objective was to determine the effect of sugar molecules on the viscosity of galactomannan solutions, in order to attempt to correlate alterations in sugar uptake with variations in viscosity of the test solutions.

4.2 Materials and methods

4.2.1 Materials

Galactomannan was extracted from fenugreek seeds (Amber variety) in our laboratory using the fraction A method, as previously described (see chapter 2 for the method to obtain fraction A). Seed coat fraction (0.5 mm mesh) was dispersed in RO water, at a ratio of 1:40 (w/v) for 4 hours. After centrifugation at 17,700 x g for 30 min, the supernatant was mixed with absolute ethanol at a ratio of 1:1 (v/v) to precipitate the fenugreek gum, which contained 91.4% (w/w) galactomannan on a dry weight basis.

(^{14}C) Carbon and (^{3}H) inulin were obtained from New England Nuclear, Boston, MA. The radiolabeled inulin (molecular weight ~5,000) was used as a nonpermeant marker of the adherent mucosal fluid volume. All other compounds were of reagent grade and were obtained from Fisher Scientific Co Ltd, Edmonton, Alberta.

4.2.2 Animals

Lean (+/+) and obese (*cp/cp*) JCR rats (4 month olds) were provided by Dr. J.C. Russell and Dr. S.D. Proctor, Faculty of Agricultural, Food and Nutritional Science, University of Alberta. Animals were fed *ad libitum* with Laboratory Autoclavable Rodent Diet (5010) containing 4.5% (w/w) fat (WF Fisher and Son, Inc). Animals were fasted for 16 hours, and were then anaesthetized by 3.5% (v/v) isoflurane/O_2. Heart exsanguination was performed before rapidly collecting the small intestine. Lean JCR rats weighing 305-350 g (n=8), and obese JCR rats weighing 500-622 g (n=8) were used. The experimental protocol was approved by the Faculty of Agriculture, Forestry and

Home Economics' Animal Policy and Welfare Committee (FAPWC), University of Alberta, Canada.

4.2.3 Preparation of incubation solutions

[^{3}H] Inulin and the 2 mM and 32 mM glucose labelled with ^{14}C-probe were prepared in oxygenated Krebs bicarbonate (pH 7.4). The buffer solutions were prepared at two fold of the final concentrations in order to be combined with galactomannan solutions the next morning at the ratio of 1:1.

4.2.4 Preparation of galactomannan solutions

Galactomannan solutions were prepared at 0.2%, 0.5%, 0.7%, and 1% (w/w) and were then diluted with buffer solution to a ratio of 1:1. The final concentration of galactomannan in the test solutions was 0.1%, 0.25%, 0.35%, and 0.5% (w/w), respectively. Galactomannan solutions were prepared on a weight for weight basis using RO water in a boiling water bath for 30 min, with gentle stirring to ensure homogeneity. The solutions were kept at 4 °C in a refrigerator overnight, with gentle stirring to ensure homogeneity and to prevent microbial growth. A correction was later made for any loss of water due to evaporation.

4.2.5 Preparation of fructose and sucrose solutions

For viscosity measurements, two solutions containing fructose or sucrose were made by the following method described in 4.2.3-4.2.4. However, there were no radioactive isotopes in the Krebs buffer. The final concentration of test solutions

contained 0.5% (w/v) galactomannan Krebs buffer which contained fructose (2 or 32 mM) or sucrose (2 or 32 mM).

4.2.6 Tissue preparation

Animals were killed by using isoflurane and cardiac exsanguination. A 15 cm length of the proximal jejunum and distal ileum were rapidly removed and rinsed gently with 50 ml of cold saline, as described in detail elsewhere (Thomson, 1980). The intestine was cut along its mesenteric border, and the mucosal surface was carefully washed with a stream of cold saline from a syringe to remove visible mucus and debris. Circular discs of the intestine were cut from a segment, mounted as a flat sheet in incubation chambers, which contained oxygenated Krebs-bicarbonate buffer (pH 7.4) at 37°C. The discs were preincubated for 10 min to allow the tissue to equilibrate at this temperature. The galactomannan solution and preincubation solutions were mixed at identical stirring rates with circular magnetic bars, and the stirring rates were precisely adjusted by means of a strobe light. The stirring rate of 600 rpm was selected to achieve low effective resistance of the intestinal unstirred water layer (UWL) (Westergaard and Dietschy, 1974).

4.2.7 Determination of glucose uptake rates

After preincubation, the chambers were transferred to other beakers containing [^{3}H] inulin and the various ^{14}C-labelled glucose solutions in oxygenated Krebs bicarbonate (pH 7.4) at 37°C. After incubation of the discs in radioactive-labelled solutions for 6 min, the experiment was terminated by removing the chamber and quickly

rinsing the tissue with cold saline for 5 seconds. The exposed mucosal tissue was then cut out of the chamber with a circular steel punch and was gently blotted on filter paper. The tissue was dried overnight in an oven at 75°C. The dry weight of the tissue was determined. Scintillation fluid was then added to the tissue saponified with 0.75 N NaOH, and the radioactivity in the tissue was determined by means of an external standardization technique to correct for variable quenching of the two isotopes. The rate of glucose uptake was expressed as nanomoles/100 mg dry weight/ minute (Westergaard and Dietschy, 1974).

4.2.8 Viscosity measurements

Viscosity measurements of galactomannan solutions were recorded at consecutive fixed shear rates of 1.29-129 s^{-1}, using a Searle type rheometer Rheolab MC1 (Physica Messtechnik Gmbit, Stuttgart, Germany), and the UDS200 program. The viscometer was equipped with a Peltier heating system that controlled the sample temperature. All viscosity measurements were recorded at 37 °C using DG 27 cup and bob geometry with a sample size of 7 ± 0.005 g. The shear rate was reported in s^{-1} after multiplying the rpm value by a conversion factor of 1.29 s^{-1} provided by the manufacturer.

4.2.9 Statistical Analyses

4.2.9.1 Rate of glucose uptake

Rate of glucose uptake was analyzed using a 3-way ANOVA comparing galactomannan concentrations, glucose concentrations, and type of rats on glucose uptake

in the jejunum and ileum. Analyses were performed using the SAS program (version 9.1, Cary, NC, USA) according to the model:

$$Y_{ijk} = \mu + GAL_i + G_j + A_k + (G*GAL)_{ij} + (G*A)_{ik} + (GAL*A)_{jk} + E_{ijk}$$

Where: Y = glucose uptake rates in either jejunum or ileum;

μ = the true mean;

GAL_i = galactomannan effect, i = 0%, 0.1%, 0.25%, 0.35%, and 0.5% (w/w);

Gj = glucose effect, j = 2 and 32 mM glucose;

A_k = type of animal effect, k = lean and obese JCR rats;

$(G*GAL)_{ij}$ $(G*A)_{ik}$ $(GAL*A)_{jk}$ = the treatment interaction

Data were expressed as least square means and were compared using probability of differences (pdiff) (SAS Institute, Inc. 2003). Statistical significance was reached with a p-value of <0.05.

4.2.9.2 Intestinal characteristics and viscosity

Data of tissue characteristics and viscosity of solutions were reported as the mean ± SEM. Characteristics of jejunum and ileum tissues were statistically analyzed by ANOVA. Statistical analyses of viscosity of solutions were calculated by repeated measurement analysis, followed by SNK as a post-hoc test. The level of $p< 0.05$ was regarded to be statistically significant.

4.3 Results

4.3.1 Characteristics of animals

This study compared the effect of various galactomannan concentrations on glucose uptake in intestinal discs from lean JCR (+/+) and obese JCR (*cp/cp*) rats. Lean JCR rats were used as a control compared to obese JCR rats. There were statistically significant differences ($p<0.001$) in body weight between the lean (326.9 ± 14.7 g) and obese rats (568.5 ± 14.7 g). However, the weight of the intestinal mucosa and submucosa was not different between the lean and obese rats ($p>0.05$, Table 4-1).

4.3.2 Effect of genotype of animal on glucose uptake

Glucose uptake into the jejunum or ileum was not statistically different ($p>0.05$) between the lean and obese rats (Appendix 6 and 7). For this reason, data from the lean and obese rats were combined in the glucose uptake experiments (Fig. 4-1 and Fig. 4-2).

4.3.3 Effect of galactomannan concentrations on glucose uptake

At 2 mM glucose, jejunal glucose uptake was significantly reduced by solutions containing 0.25%, 0.35%, and 0.5% (w/w) galactomannan compared to control solution containing no galactomannan ($p <0.01$, Fig. 4-1). At 32 mM glucose, jejunal glucose uptake was significantly reduced by 0.1%, 0.25%, and 0.35% (w/w) galactomannan solutions compared to the control solution ($p <0.01$).

In the ileum (Appendix 7), there were significant interactions between galactomannan and glucose concentrations, and between glucose concentrations and genotype of rats. Therefore, these parameters (e.g. galactomannan and glucose) could not

be considered separately. At 2 mM, glucose uptake was significantly reduced by solutions containing 0.35% and 0.5% (w/w) galactomannan in both the lean and obese rats compared to the control (Fig 4-2). At 32 mM, glucose uptake was reduced in the presence of 0.1%, 0.25%, 0.35% of galactomannan but not of 0.5% (w/w) galactomannan.

4.3.4 Viscosity of galactomannan containing sugar solutions

The value for the viscosity of all solutions used in this study was presented as apparent viscosity, due to their pseudo-plastic behaviour observed in the preliminary study (chapter 3).

At 2 mM glucose, the viscosity of 0.1% and 0.25% (w/w) galactomannan solutions at low shear rate (1.29, 1/s) was significantly reduced after stirring for 60 min as compared to 0 min ($p < 0.05$, Table 4-2). In contrast, the viscosity of 0.35% and 0.5% (w/w) galactomannan solutions was increased after stirring for 60 min when compared to 0 min ($p < 0.05$).

At 32 mM glucose, the viscosity of the control, 0.1% and 0.25% (w/w) galactomannan solutions was not significantly different after stirring for 60 min as compared to 0 min (Table 4-3). In contrast, the viscosity of 0.35% and 0.5% (w/w) galactomannan solutions was increased after stirring for 60 min when compared to 0 min ($p < 0.05$).

The viscosity of sucrose and fructose solutions was determined in order to investigate the interaction of 0.5% (w/w) galactomannan with another monosaccharide (2 or 32 mM fructose) or disaccharide (2 or 32 mM sucrose). This, in turn, could provide

some information (i.e. the expansion of galactomannan network) related to the interaction of 0.5% (w/w) galactomannan and glucose molecule which was focused in the present study. The viscosity of 0.5% (w/w) galactomannan mixed with 32 mM sucrose was significantly greater than that of 0.5% (w/w) galactomannan mixed with 2 mM sucrose in all shear rate measurements ($p<0.05$, Table 4-4). However, the viscosity of the 0.5% (w/w) galactomannan solution mixed with either 2 or 32 mM fructose was not significantly different in all shear rate measurements ($p>0.05$, Table 4-5).

4.4 Discussion

Previous studies have indicated that viscous polysaccharides may inhibit intestinal absorption of nutrients by increasing the apparent thickness of the UWL, through which nutrients must diffuse before reaching the mucosal surface (Elsenhan *et al.*, 1980; Johnson and Gee 1981). Johnson and Gee (1981) observed that incubation of the small intestine for 15 minutes in a medium containing 0.5 % (w/w) guar gum led to an increase in the apparent thickness of the UWL by 48% and to a decrease in the rate of glucose uptake by 74% when compared with sacs preincubated without guar gum. Furthermore, in the presence of high UWL resistance caused by guar solution, the value of the apparent Michaelis constant (K_m^*) was increased without changing the calculated maximal transport rate (Vmax) (Elsenhans *et al.*, 1984). These observations were in agreement with other researchers who reported that viscous fiber solutions inhibit glucose uptake with an increase in the Km, and unaltered Vmax (Winne, 1973; Wilson and Dietschy, 1974). Experimentally (Lewis and Fordtran, 1975; Thomson and Dietschy, 1980; Elsenhan *et al.*,1984) and theoretically (Wilson and Dietschy, 1974) it can be shown that

the expected effect of increased viscosity of the test solution is an increase of the value of the Km without effecting the value of the Vmax, so that the uptake of low (2 mM) but not high (32 mM) concentrations of solute would be reclaimed. In the present study, the 2 mM glucose was chosen because the Km for D-glucose uptake was 1-2 mM (Thomson, 1986). Any change of the rate of glucose uptake in a 2 mM glucose-containing solution will reflect the effect of the increase of the UWL resistance.

At 2 mM glucose, 0.25-0.5% (w/w) galactomannan-containing solutions significantly reduced the glucose uptake rate as compared to the control in the jejunum (Fig 4-1). Meanwhile, 0.35-0.5% (w/w) galactomannan-containing solutions significantly reduced glucose uptake rate compared to the control in the ileum (Fig 4-2). The inhibitory effect of galactomannan on glucose uptake in the present study was due to increase in viscosity of galactomannan solution. The viscosity of 0.1%, 0.25%, 0.35%, and 0.5% (w/w) galactomannan solutions after stirring for 60 min was 3, 10, 41, and 268 folds higher than that of control, respectively (Table 4-2). However, we could not see any further decrease in the rate of glucose uptake between 0.35% and 0.5% (w/w) galactomannan solutions. This trend could be explained by looking at the mass transfer behaviour of glucose in a viscous layer before reaching the mucosal surface. Mass transfer partly depends on the diffusion coefficient, which is a function of viscosity (Knudsen *et al.*, 1997). According to the Stokes-Einstein equation of diffusivity estimation in liquid (Appendix 8), the diffusion coefficient is indirectly proportional to the viscosity. As viscosity increases the effect of lowering the diffusion coefficient is reduced. Therefore, it is most likely that the diffusion of glucose through either 0.35% or 0.5% (w/w) galactomannan solution layer was similar. Our results suggest that

galactomannan solution at 0.35% (w/w) was the optimal content to decrease the rate of glucose uptake in both the jejunum and ileum. Thus, we know that the galactomannan-containing solutions increased the viscosity of the bulk phase solutions (Johnson and Gee, 1981), the increase in viscosity would increase the value of the apparent Km (Winne, 1973; Wilson and Dietschy, 1974), and the increase in the value of the apparent Km would reduce the rate of uptake of glucose at concentrations of glucose near the value of the apparent Km (i.e., 2 mM glucose, as used in this study).

Galactomannan is a polysaccharide polymer that dissolves in water because of the presence of galactose side chain. Due to its hydrophilic nature, hydrogen bonds are formed between galactose and water, creating network formations by the entanglement of fully hydrated chains of galactomannan; this produces viscous solutions (Cybulska and Doe, 2002). As shown in Fig. 4-1 and 4-2, the rate of glucose uptake in the presence of 2 mM glucose was negatively and linearly correlated with galactomannan concentrations in the range 0.1%-0.35% (w/w) in the jejunum (R^2 = 21.3%, $p < 0.001$, Appendix 9), and in the ileum (R^2 = 13.7%, $p < 0.001$, Appendix 10). These results suggest that the reduced rate of glucose uptake in the small intestine may be due to the ability of the galactomannan to act as a significant physical barrier to glucose diffusion.

At concentration at least 5-times above the value of the Km, glucose uptake will be a reflection of the value of the Vmax. Thus, the 32 mM concentration of glucose was used to assess possible effects of galactomannan on the reduction of the Vmax. It should be noted that this value, 32 mM, is clearly within the range of glucose concentration in the lumen of the rat intestine after meals (Ferraris *et al.*, 1990). Theoretically, changes in the thickness of the UWL have no effect on the value of the Vmax (Wilson and Dietschy,

1974). However, our results suggest the converse, since 0.1%-0.35% (w/w) galactomannan-containing solutions significantly reduced rate of glucose uptake compared to the control in both the jejunum (Fig 4-1) and ileum (Fig 4-2) and the rate of glucose uptake was shown in non-linear relation with galactomannan concentrations in both the jejunum and ileum (Appendices 11 and 12). These may reflect the significance of the thickness of galactomannan in the bulk phase affecting a high resistance diffusion of the probe molecule to the mucosal surface. However, this effect had not been seen in 0.5% galactomannan mixed with 32 mM glucose solution. This point requires further study and will be discussed below. The negative inhibitory effect of 0.5% (w/w) galactomannan solution on 32 mM glucose in our study was in agreement with Johnson and Gee (1981), who studied the effect of various guar gum concentrations on glucose uptake in the presence of 28 mM glucose concentration. They reported that 0.1% and 0.25% (w/w) guar gum concentrations showed a significant reduction in jejunal glucose uptake by 10 and 21 μmol/g/30 min compared to control group, respectively. However, 0.5% (w/w) guar gum concentration had similar reduction in glucose uptake as the 0.25% (w/w) guar gum group (Johnson and Gee, 1981).

The value of the measurement on apparent Vmax (Vmax*) is comprised of the Vmax of SGLT-1 in the brush border membrane (BBM), the Vmax of GLUT 2 in both the BBM and basolateral membrane (BLM), and the contribution of the passive component of glucose uptake (as can be estimated from the rate of uptake of L-glucose). The inhibitory effect of galactomannan on the uptake of 32 mM glucose in the present study (Fig 4-1 and 4-2) could be explained in part by the reduction in the passive component due to the increasing viscosity of the solution enhancing the value of the

UWL. However, at the highest concentration of galactomannan (0.5%, w/w), the uptake of 32 mM was higher than that of 0.25% and 0.35% (w/w, Fig 4-1 and 4-2). We speculate that the higher concentrations of galactomannan actually enhance the uptake of glucose mediated by SGLT-1 and GLUT 2. If this is the case, then the amount of galactomannan to be administered (and therefore its concentration in the intestinal lumen) must be carefully adjusted to achieve a specific outcome: if it is desired to use galactomannan to inhibit uptake at low concentrations of glucose, a range of galactomannan 0.25-0.35% (w/w) may be used. In contrast, if galactomannan is administered with the intent of reducing the uptake of higher concentrations of glucose, the amount of galactomannan ingested and its concentration in the intestinal lumen must not be high, since this would result in a paradoxical effect.

Since the viscosity of viscous polysaccharides play an important role on inhibitory of glucose uptake (Johnson and Gee, 1981; Kim, 2005), a continuous decline in the rate of glucose uptake is expected as the concentration of galactomannan increases. As this was not the case in the presence of 32 mM glucose, we have attempted to gain a probable explanation for this result by measuring the viscosity of the tested solutions. It was shown that viscosity increased with increasing concentrations of galactomannan (Table 4-2 and 4-3). Interestingly, we found that the viscosity of 0.5% (w/w) galactomannan mixed with 32 mM glucose solution (Table 4-3) was significantly less than the viscosity of 0.5% (w/w) galactomannan mixed with 2 mM glucose (Table 4-2). This may explain the influence of glucose molecule (between 2 mM and 32 mM) on the inhibitory effect of glucose uptake of 0.5% (w/w) galactomannan as shown in Fig 4-1 and 4-2. We speculate that when 0.5% (w/w) galactomannan was mixed with 32 mM glucose,

the high concentration of glucose molecules may interfere with the formation of galactomannan networks, and then reduce the viscosity of galactomannan solution.

In order to examine whether other sugar molecules interfere with galactomannan network, sucrose (2 and 32 mM) or fructose (2 and 32 mM) mixed with 0.5% (w/w) galactomannan solutions were assessed for their viscosity using the same conditions as described for glucose. The viscosity of 32 mM sucrose mixed with 0.5% (w/w) galactomannan was higher than the solution of 2 mM sucrose mixed with 0.5% (w/w) galactomannan (Table 4-4). Contrary to sucrose, the viscosity of 32 mM fructose mixed with 0.5% (w/w) galactomannan was similar to that of 2 mM fructose mixed with 0.5% (w/w) galactomannan (Table 4-5). Monosaccharides, such as glucose and fructose are hydrophilic molecules that strongly interact with water (Cybulska and Doe, 2002). Our results suggest that high concentrations of monosaccharide (32 mM) reduce the availability of water in the solution preventing the expansion of the galactomannan networks, thereby decreasing the viscosity. Consistent with this hypothesis, it was reported that added sugars restricted the hydration of guar gum in the solution (Elfak et al., 1977; Doyle et al., 2006).

In the present study, the glucose solution was mixed with galactomannan solution and then stirred for 60 minutes to ensure homogeneity before determination of the rate of glucose uptake in the small intestine. We therefore determined whether time (0, 30, and 60 min) affect the viscosity of tested solutions. Our results showed that in solutions containing 2 mM glucose with low galactomannan concentrations (either at 0.1% or 0.25%, w/w), the viscosity was reduced after stirring the solution for 60 min (Table 4-2). These effects, however, were not seen in the solution of 2 mM glucose mixed with high

galactomannan concentrations (0.35% and 0.5%, w/w). These results suggest that the expansion of galactomannan in solution increases with time. It is possible that glucose molecules trapped in the network of galactomannan may change its structure and reduce the viscosity. However, in the presence of high galactomannan concentrations (0.35% and 0.5%, w/w), the excess galactomannan molecules (without trapped glucose in the network) expand and create networks that increase the viscosity of the solution. These tendencies are similar in the presence of 32 mM glucose.

In the present study, we did not see any difference in the rate of glucose uptake between lean and obese JCR rats. (Appendix 6 and 7). Based on our knowledge, glucose uptake in the intestine has not been previously studied in the JCR rat. Factors that contribute to obesity in the JCR (LA: *cp/cp*) rats are: i) they are hyperphagic and consume twice the food of lean JCR rats (Dr. J.C.Russell, personal communication), ii) they have a longer small intestine (Dr. J.C. Russell, unpublished data). The body weight of the obese JCR (LA: *cp/cp*) rats used in the present study was, as expected, about 60% greater than the lean control rats (568.5 ± 14.7 vs 326.9 ± 14.7 g, $p<0.001$). The weight of the intestine bears a rough correlation with the weight of the animal (Ferraris and Diamond, 1997), and the length of the intestine of the obese JCR rats is greater than in the lean controls (Dr. J.C. Russell, unpublished data). However, there was no variation between these two groups in the weight of the mucosa, submucosa, or % of the intestinal wall comprised of mucosa (Table 4-1). This observation has several important implications. Firstly, we did not assess the morphology of the intestine in these animals, but the similarity in the mucosa weight suggests that this is unlikely to be any major difference in the weight of the intestinal villi or mucosal surface area. Secondly, the

predicted implication of this is therefore the lack of need to express the glucose uptake results on the weight of the mucosa: expressing uptake on the basis of the weight of the intestine (nmol/100mg/min), as was done here, is reasonable. Thirdly, although the genotype of rat (obese vs lean) did not affect glucose uptake, it may be speculated that the total uptake of glucose in the obese rats would be greater than in the control animals, because of the greater length of their intestines. Finally, the food intake in the obese rats was greater than in the lean controls (34.2 ± 1.4 vs 19.5 ± 0.9 g/day, $p<0.001$).

Another factor that contribute to obesity in the JCR (LA: *cp/cp*) rat is due to lack of leptin receptors which contribute to hyperphagia. The *cp* gene has been shown to be a stop codon in the extracellular domain of the leptin receptor (ObR). This leads to a complete absence of the ObR in the plasma membrane of the *cp/cp* animals (Russell, 2001). Recently, the studies have shown that the leptin receptor is present in stomach and intestinal mucosa (Barrenetxe *et al.*, 2002, Cammisotto *et al.*, 2006). Luminal leptin binds to apical leptin receptor on jejunum enterocytes to decrease the activity of SGLT-1. The mechanisms that are responsible for leptin inhibition of SGLT-1 function may involve modulation of its intrinsic activity and/or abundance of SGLT-1 proteins in the BBM. This suggested that leptin effects involve a decreased insertion of SGLT-1 molecules recruited from the preformed intracellular pool into the BBM (Ducroc *et al.*, 2005). Our results showed that there was no difference in the rate of glucose uptake between lean and obese JCR rats, suggesting that the activity of SGLT-1 proteins in the BBM of obese JCR rats may not have been altered. However, this need to be further elucidated.

Considering the non-significant difference of the rate of glucose uptake between lean and obese JCR rats, one may query with the method used in this study. We would like to provide the reasons of choosing intestinal disc (*in vitro*) method and incubation time in the present study.

Tissue preparation is one of the factors, which correctly characterize an active intestinal transport process. Thomson and Dietschy (1980) indicated that three preparations of *in vitro* tissue (intestinal discs mounted in a chamber, everted jejunal sacs, and full-thickness jejunal biopsies) had different values of apparent permeability coefficient (Pd*). The Pd* of D-glucose was considerably lower in intestinal disc than in everted sacs or intestinal biopsies. Everted jejunal sacs and biopsy preparation may be related to variable effects on intercellular junctions, to the transport and metabolic functions of tissues other than the BBM, respectively. It is likely that use of disc represented for solely BBM carrier for D-glucose was appropriate in our study.

The selection of incubation times in the present study was justified based on previous research. Theoretically, the incubation should be long enough to allow complete equilibration of the adherent fluid with both glucose and inulin in the bathing solution, thereby permitting an accurate correction for glucose in the adherent fluid (Ferraris and Diamond, 1986). If there was a failure of the marker to equilibrate, the uptake of glucose at 1 and 2 min could have been overestimated by at least 100% (Thomson and Dietschy, 1980a). The incubation also should be brief enough that uptake of the glucose is still linear with time, and that loss of counts to the serosal surface is negligible (Solberg and Diamond, 1987). It was shown that incubation times of 6 to 10 minutes are appropriate under most conditions of stirring and for most solutes (Lukie *et al.*, 1974). Time less

than 6 minutes is insufficient to allow complete equilibration of the extracellular volume marker with the unstirred layer, and may cause overestimation of uptake rates. Time more than 10 minutes results in underestimation of flux rates due to loss of the labeled probe molecule into the serosal compartment (Lukie *et al.*, 1974). In the present study, although we did not test a time trial in a preliminary study, based on previous studies in our laboratory incubation for 6 minutes was an optimal for the glucose uptake study (Reimer *et al.*, 1997; Drozdowski *et al.*, 2003).

In the present study, inulin was used as an extracellular fluid marker to calculate the adherent mucosal fluid volume. Total uptake rate can be broken down into two components: specific transport and diffusion into the extracellular space (Alvarado *et al.*, 1984). The aqueous diffusion barrier in the extracellular space may cause marked alterations in the kinetics of active transport (Westergaard and Dietschy, 1974). Therefore, total specific transport should be corrected for diffusion by using the appropriate extracellular marker. Thomson and Dietschy (1980a) made this correction by using ^{3}H dextran (as an adherent fluid marker) and ^{14}C D-glucose to measure total uptake in one tissue, then using ^{3}H dextran and ^{14}C L-glucose to measure passive uptake in another tissue. L-glucose uptake at the concentration of D-glucose was calculated on the assumption that the uptake vs concentration relation for L-glucose is linear. Subtraction of this L-glucose uptake from D-glucose uptake corrects simultaneously for adherent fluid and passive uptake, yielding active uptake directly.

An ideal marker of adherent fluid volume should be totally excluded from the cell and should not be susceptible to metabolic or physical alteration during the course of a study (Sallee *et al.*, 1972; Alvarado *et al.*, 1984). Inulin is suitable for assessing the

diffusion of molecules because of physical properties. It is high water solubility and non-permeant molecule (MW 5,000-5,500). A comparison of using 5 nonpermeant markers in the same intestinal segment showed that [^{3}H]inulin yield adherent mucosal fluid volumes as much as 20% greater than [^{3}H] dextran, [^{14}C] dextran, [^{14}C] polyethylene glycol, and [^{14}C]inulin. This may due to rapid exchange of labeled tritium with tissue protons (Sallee *et al.*, 1972).

In summary, we identified two factors that influenced the rate of glucose uptake: galactomannan and glucose concentrations. At 2 mM glucose, adding 0.25% to 0.5% (w/w) galactomannan reduced glucose uptake in the jejunum. Adding 0.1% to 0.35% (w/w) galactomannan in 32 mM glucose solution reduced the uptake of glucose in the jejunum. Similar trends were observed in the ileum. Under these conditions, the genotype of rats (lean vs. obese rats) did not influence the rate of glucose uptake in the jejunum. Therefore, galactomannan and glucose influenced the viscosity of the solution and the uptake of glucose in the small intestine.

Our results suggest that the concentrations of galactomannan and glucose affected the viscosity of the solution. Specifically, viscosity increased with increasing concentrations of galactomannan. Glucose at 32 mM concentration had a high affinity for water molecules preventing the expansion of the network in the 0.5% (w/w) galactomannan solution. Thus, the development of intestinal viscosity and the transport of glucose through intestinal mucosa were relatively dependent on the network formation of galactomannan in the solution.

Table 4-1. Tissue characteristics of lean and obese JCR rats

Animals	Jejunum			Ileum		
	Mucosa (mg/cm)	Submucosa (mg/cm)	% Mucosa	Mucosa (mg/cm)	Submucosa (mg/cm)	% Mucosa
Lean rat	11.0±1.3	1.3±0.2	86.7±2.9	7.5±1.3	1.3±0.2	83.1±2.8
Obese rat	10.8±1.3	1.4±0.2	86.9±2.9	9.3±1.3	1.2±0.2	87.8±2.8

Value is mean ± SEM., n = 8/group

None of the differences were statistically significant between lean and obese rats within the same column ($p > 0.05$).

Table 4-2. Viscosity of various galactomannan solutions mixed with 2 mM glucose after stirring for 0, 30 and 60 minutes

Galactomannan concentration (%, w/w)	Time (min)	Shear rate [1/s]					
		1.29	6.46	12.9	25.8	64.6	129
Control (0%)	0	0.9±0.3	0.9±0.04	0.9±0.04	0.8±0.04	0.9±0.04	1.1±0.07
	30	0.8±0.3	0.8±0.04	0.8±0.04	0.7±0.04	0.7±0.04	1.0±0.07
	60	0.8±0.3	0.7±0.04	0.7±0.04	0.7±0.04	0.8±0.04	1.0±0.07
0.1%	0	9.5±0.1^{a}	3.7±0.1^{a}	2.8±0.1^{a}	2.3±0.1	2.0±0.1	1.9±0.1
	30	3.3±0.1^{b}	2.3±0.1^{b}	2±0.1^{b}	1.9±0.1	1.8±0.1	1.8±0.1
	60	2.4±0.1^{c}	1.9±0.1^{c}	1.8±0.1^{b}	1.8±0.1	1.8±0.1	1.8±0.1
0.25%	0	9.4±0.3^{a}	6.5±0.2	5.6±0.2^{b}	5.2±0.2^{b}	4.8±0.2^{b}	4.5±0.4
	30	6.6±0.3^{b}	5.9±0.2	5.6±0.2^{b}	5.5±0.2^{b}	5.2±0.2^{b}	4.9±0.4
	60	7.9±0.3^{b}	6.9±0.2	6.6±0.2^{a}	6.4±0.2^{a}	6.0±0.2^{a}	5.6±0.4
0.35%	0	27±0.7^{b}	21±0.4^{b}	19±0.4^{b}	17±0.4^{b}	15±0.9	13±0.6^{b}
	30	25±0.7^{b}	21±0.4^{b}	20±0.4^{b}	18±0.4^{b}	16±0.9	14±0.6^{b}
	60	33±0.7^{a}	27±0.4^{a}	25±0.4^{a}	23±0.4^{a}	19±0.9	17±0.6^{a}
0.5%	0	173±2.1^{b}	131±2.0^{b}	111±2.4^{b}	89±1.1^{b}	63±1.1^{b}	46±0.7^{b}
	30	171±2.1^{b}	128±2.0^{b}	108±2.4^{b}	88±1.1^{b}	62±1.1^{b}	46±0.7^{b}
	60	214±2.1^{a}	156±2.0^{a}	130±2.4^{a}	104±1.1^{a}	72±1.1^{a}	52±0.7^{a}

Value is mean ± SEM, n=4
Unit of apparent viscosity is milli pascal second (mPa·s)
$^{a-c}$ Different letters following means at 0, 30, and 60 min within the same column of each galactomannan concentration indicate a significant difference at the p <0.05 level.

Table 4-3. Viscosity of various galactomannan solutions mixed with 32 mM glucose after stirring for 0, 30 and 60 minutes

Galactomannan concentration (%, w/w)	Time (min)	Shear rate [1/s]					
		1.29	6.46	12.9	25.8	64.6	129
Control (0%)	0	1.0±0.1	0.9±0.05	0.9±0.05	0.9±0.05	0.9±0.05	1.1±0.07
	30	0.9±0.1	0.8±0.05	0.8±0.05	0.8±0.05	0.8±0.05	1.1±0.07
	60	0.9±0.1	0.8±0.05	0.8±0.05	0.8±0.05	0.8±0.05	1.1±0.07
0.1%	0	1.8±0.1	1.8±0.1	1.8±0.1	1.7±0.2	1.8±0.2	1.8±0.2
	30	1.9±0.1	1.9±0.1	1.9±0.1	1.9±0.2	1.9±0.2	1.9±0.2
	60	2.0±0.1	2.0±0.1	2.0±0.1	2.0±0.2	2.0±0.2	2.0±0.2
0.25%	0	4.8±0.4	4.8±0.4	4.7±0.4	4.6±0.4	4.5±0.3	4.3±0.3
	30	5.9±0.4	5.5±0.4	5.4±0.4	5.2±0.4	5.0±0.3	4.7±0.3
	60	6.7±0.4	6.0±0.4	5.8±0.4	5.6±0.4	5.3±0.3	5.1±0.3
0.35%	0	22 ± 1.0^{c}	19 ± 1.1^{b}	18 ± 0.7^{c}	16 ± 0.9^{b}	14±1.1	13 ± 0.7^{b}
	30	27 ± 1.0^{b}	23 ± 1.1^{ab}	21 ± 0.7^{b}	19 ± 0.9^{ab}	17±1.1	14 ± 0.7^{ab}
	60	32 ± 1.0^{a}	26 ± 1.1^{a}	24 ± 0.7^{a}	22 ± 0.9^{a}	19±1.1	16 ± 0.7^{a}
0.5%	0	92 ± 1.4^{c}	74 ± 1.2^{c}	65 ± 1.4^{c}	55 ± 1.0^{c}	41 ± 0.8^{c}	32 ± 0.9^{b}
	30	102 ± 1.4^{b}	80 ± 1.2^{b}	70 ± 1.4^{b}	60 ± 1.0^{b}	45 ± 0.8^{b}	34 ± 0.9^{ab}
	60	123 ± 1.4^{a}	97 ± 1.2^{a}	84 ± 1.4^{a}	70 ± 1.0^{a}	52 ± 0.8^{a}	39 ± 0.9^{a}

Value is mean ± SEM, n=4
Unit of apparent viscosity is milli pascal second (mPa·s)
[a-c] Different letters following means at 0, 30, and 60 min within the same column of each galactomannan concentration indicate a significant difference at the $p < 0.05$ level.

Table4-4.Viscosity of 0.5% (w/w) galactomannan solution mixed with either 2 or 32 mM sucrose after stirring for 60 minutes

Sucrose	Shear rate [1/s]					
	1.29	6.46	12.9	25.8	64.6	129
2 mM	146±1.1[b]	103±1.1[b]	85.1±1.0[b]	67.5±1.1[b]	46.4±0.9[b]	32.7±0.7[b]
32 mM	241±0.9[a]	165±1.1[a]	135±1.0[a]	106±0.7[a]	71.5±0.9[a]	49.8±1.6[a]

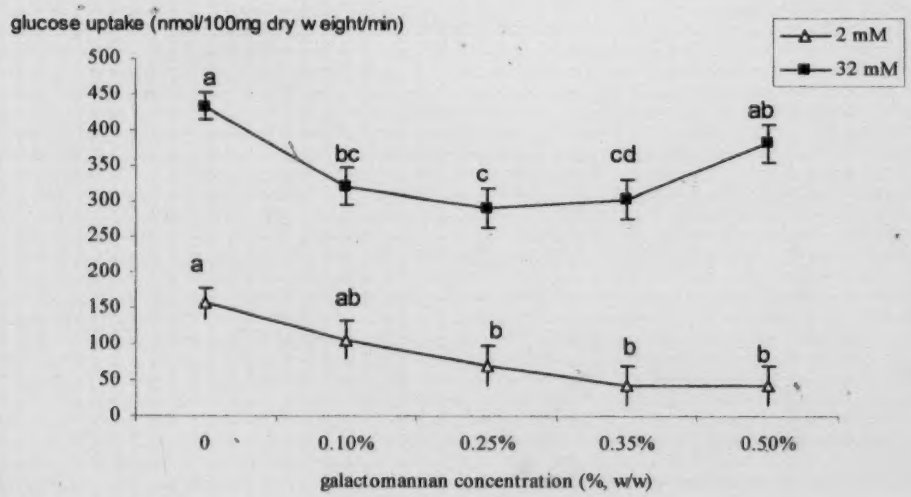
Value is mean ± SEM, n=4
Unit of apparent viscosity is milli pascal second (mPa·s)
[a-b] Different letters following means within the same column indicate a significant difference at the p <0.05 level.

Table4-5.Viscosity of 0.5% (w/w) galactomannan solution mixed with either 2 or 32 mM fructose after stirring for 60 minutes

Fructose	Shear rate [1/s]					
	1.29	6.46	12.9	25.8	64.6	129
2 mM	103.0±0.9	79.8±1.8	69.3±1.3	58.4±1.1	44.0±1.6	33.6±0.9
32 mM	103.0±1.4	78.1±1.1	67.3±1.7	56.4±1.6	42.1±0.9	31.9±0.6

Value is mean ± SEM, n=4
Unit of apparent viscosity is milli pascal second (mPa·s)
There is no significant difference between means within the same column.

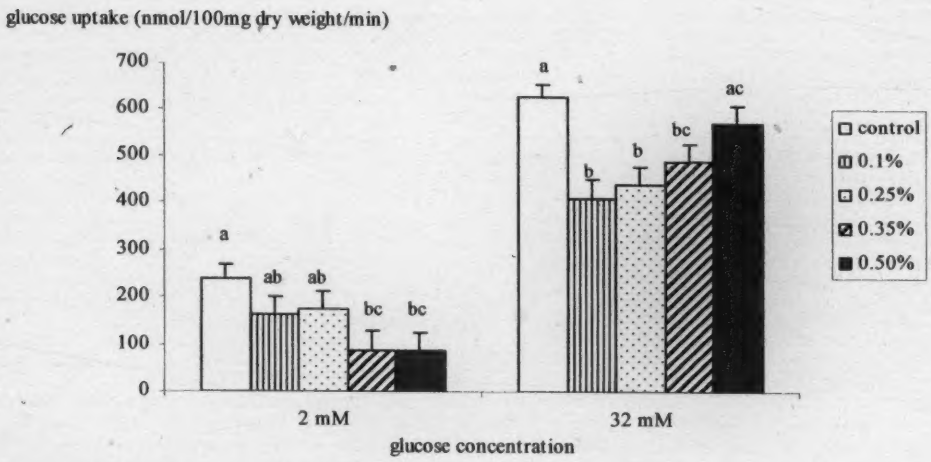
Figure 4-1. The effect of various galactomannan concentrations on glucose uptake level in the jejunum of rats in the presence of 2 mM (--Δ--) and 32 mM (■) glucose concentrations.



Each point represents the mean ± SEM of the results of 16 rats.

[a-d] Different letters following means within a line indicate a significant difference at the $p < 0.05$ level.

Figure 4-2. The effect of various galactomannan concentrations on glucose uptake level in the ileum rats in the presence of 2 and 32 mM glucose concentrations.



Each point represents the mean ± SEM of the results of 16 rats.

[a-c] Different letters following means within 2 mM or 32 mM glucose indicate a significant difference at the $p < 0.05$ level.

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Chapter 5

The modifying effects of galactomannan on the glycemic and lipidemic status in rats

5.1 Introduction

Galactomannan (i.e. fenugreek seeds, guar gum) containing diets have been shown to reduce postprandial blood glucose (Sharma, 1986) and improve insulin sensitivity in non-diabetic (Jenkins *et al.*, 1980; Fairchild *et al.*, 1996) and in diabetic individuals (Jenkins *et al.*, 1978; Sels *et al.*, 1987; Pick *et al.*, 1996). Decreasing postprandial blood glucose reduced the need for antidiabetic medication or insulin in diabetic subjects consuming diets containing guar gum. Guar-galactomannan fiber has also been shown to reduce both total and LDL cholesterol levels in healthy and T2D subjects (Jenkins *et al.*, 1980a; Landin *et al.*, 1992). However, demonstrations of such responses are not consistent among all studies. In the study by Uusitupa *et al.* (1989), guar gum-containing diets given for 4 weeks did not show an improvement in glucose control in T2D subjects. However, the chronic ingestion of guar gum has been associated with increased plasma insulin concentrations during oral glucose tolerance test (OGTT) (Groop *et al.*, 1993). Additionally, Nieuwenhoven *et al.* (2001) reported that guar gum, up to a dosage of 4.5g/200 ml, had no effect in healthy male subjects. These discrepancies may lie in the differences of galactomannan concentrations, type of food and duration of study.

The results of the intestinal glucose uptake in rats (chapter 4) showed that galactomannan had the potential to reduce glucose uptake in the intestine of JCR rats (both lean and obese). This study examined the effects of feeding galactomannan extract

(chapter 2) in a high sucrose diet on the absorption of glucose and lipid. This study was undertaken to assess the effect of an acute bolus feed of galactomannan with glucose on postprandial increase in blood glucose, and to investigate the effect of feeding galactomannan for four weeks on the glycemic and lipidemic status in healthy rats. Since a high sucrose diet has been reported to increased plasma triglyceride (TG) and TG accumulation in adipose tissue (Aghelli *et al.*, 1998), the present study was further extended to determine histologically the effect of sucrose on TG deposition in adipose tissue.

5.2 Materials and methods

5.2.1 *Glucose Load Study*

5.2.1.1 Animals and diet

Ten male Sprague-Dawley rats (weighing175-200 g) were obtained from Charles River Laboratories (Ontario, Canada). The animals were housed two per cage at 20-22 °C and subjected to a 12-h day/night cycle. They were provided *ad libitum* access to water and the AIN-93G diet (Dyets Inc, Bethlehem, PA). Animal weights and their food consumption were measured daily. The protocol was approved by the FAPWC, University of Alberta, Canada.

5.2.1.2 Oral Glucose Tolerance Test

After 1 week of acclimatization, animals were fasted for 12 hours prior to the OGTT. Each rat had three OGTT during the study. In the first week, 10 rats were tested with 75% (w/w) glucose solution (3 g/kgBW) and used as the control. In week 2,

animals were randomly separated into two groups. Group 1 (n=5) were gavaged with 75% glucose (3 g/kgBW) mixed with 0.5% (w/w) galactomannan solution. The next week (week 3) rats were gavaged with 75% glucose (3 g/kgBW) mixed with 1.0% galactomannan solution. The order of these treatments was reversed for the second group (n=5) to compensate for a potential carry-over effect. All rats had a one week of wash-out period between each bolus challenge (Fig 5-1). Rats were fasted for 12 hours prior to the oral glucose challenge. Blood samples were obtained from the tail at time 0, 15, 30, 60 and 120 min for plasma glucose determination (described below in section 5.2.2.4).

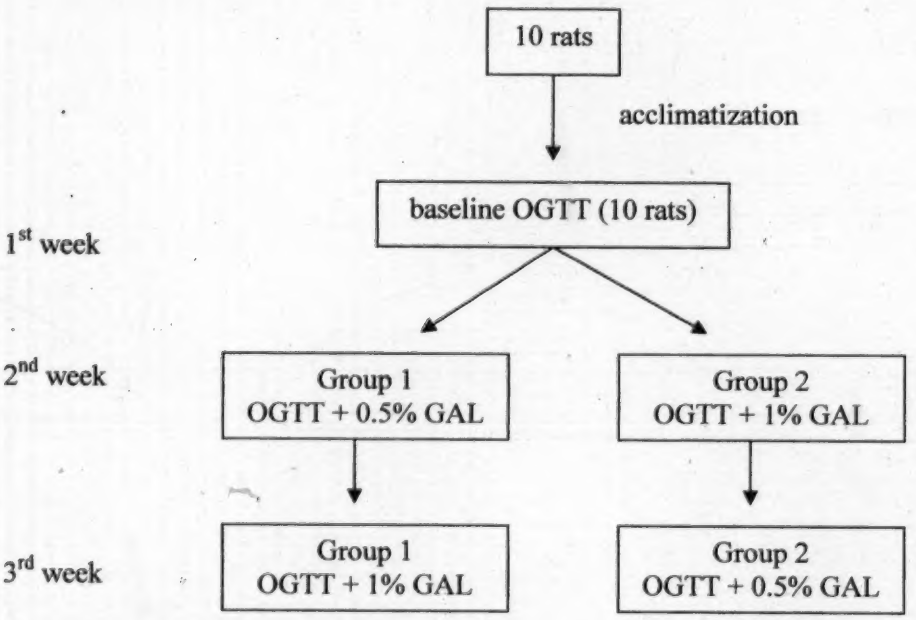


Fig 5-1. Experimental design for glucose load study. Rats were acclimatized for one week then baseline OGTT was conducted. Rats were randomly assigned to group 1 that received 0.5% galactomannan or group 2 that received 1% galactomannan during week 2. The following week, rats received the other amount of galactomannan in the third OGTT.

5.2.2 *Four- week feeding study*

5.2.2.1 Animals and diets

Twenty-five male Sprague-Dawley rats (weighing 175-200 g) were obtained from Charles River Laboratories (Ontario, Canada). The animals were housed two per cage at 20-22°C and subjected to a 12-h day/night cycle. After a week of acclimatization, animals were randomly divided into three groups: control group (n=9), 2.5%(w/w) galactomannan group, as called 'low GAL group' (n=8), and 5% (w/w) galactomannan group, as called 'high GAL group' (n=8). Initial body weights were similar among the groups. All rats had *ad libitum* access to water and food during the experiment. Among the 3 groups of animals, the diet was isocaloric and differed only in their compositions of fiber as shown in Table 5-1. Control group was fed with high sucrose diet (52%, w/w) containing 10% w/w cellulose. Low GAL and high GAL groups were fed with the control diet containing 2.5% and 5% (w/w) galactomannan, respectively, then added with cellulose to make the total dietary fiber content 10%. The total fiber content remained constant between diets. The 5% (w/w) galactomannan used in the diet was based on a reported rodent diet which contains 5% (w/w) cellulose (Artiss *et al.*, 2006). A 2.5% (w/w) galactomannan content was used in the low GAL group. Cellulose, a non-fermentable, non-soluble fiber was included in all the diets as there is a risk of diarrhea when only soluble fibers are fed. Animal weights and their food consumptions were measured daily. The protocol was approved by the FAWCP, University of Alberta, Canada.

5.2.2.2 Oral Glucose Tolerance Test

After animals were fed their respective diet for 3 weeks (Fig 5-2) and they were fasted for 12 hours prior to the OGTT. All rats were gavaged with 75% glucose solution (3 g/kgBW). Blood samples were obtained from tail at 0, 15, 30, 60, 120 min after the glucose challenge. Plasma glucose concentration was determined as described in section 5.2.2.4.

The area under the curve (AUC) of plasma glucose and plasma insulin was calculated as:

$$AUC = \tfrac{1}{2}(t_2 - t_1)(C_2 + C_1) + \tfrac{1}{2}(t_3-t_2)(C_3+C_2) + \tfrac{1}{2}(t_4-t_3)(C_4+C_3) + \tfrac{1}{2}(t_5-t_4)(C_5+C_4)$$

Where C_1, C_2, C_3, C_4 and C_5 are either plasma glucose or plasma insulin at times 0 (t_1), 15 (t_2), 30 (t_3), 60 (t_4) and 120 minutes (t_5), respectively, after glucose load.

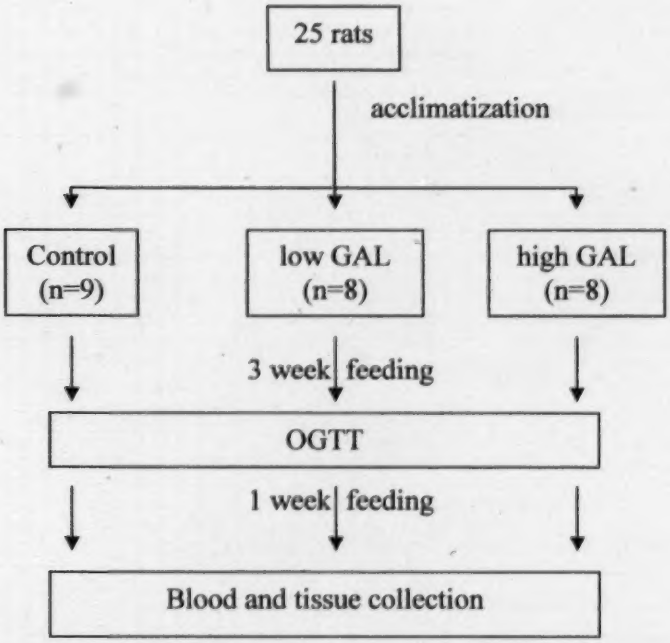


Fig 5-2. Experimental design for four-week feeding study. Rats were acclimatized for one week, then fed their respective diet for three weeks and received an OGTT. One week after the OGTT, rats were sacrificed to collect whole blood and organs.

5.2.2.3 Blood and tissue sampling

At the termination of the experiment (day 28), animals were fasted for 12 hours and anaesthetized with halothane. Cardiac puncture was performed to obtain sufficient blood for biochemical analyses. Tubes containing whole blood were centrifuged at 3000 rpm for 15 min at 4°C (Beckman J2-HC centrifuge, CA, USA). The plasma was transferred to microcentrifuge tube, which were stored at -80°C until analyzed. Death of

the rats was ensured by cervical dislocation. Liver, epididymal and peri-renal fat depots were excised, weighed, snap frozen in liquid nitrogen and stored at -80°C until analysed.

5.2.2.4 Biochemical analyses in plasma and tissue

Plasma glucose concentrations were determined by using a glucose oxidase method (Biopacific Diagnostic Inc, BC, CA). Plasma insulin was assayed using a RIA kit [^{125}I] (Linco Research, Missouri, USA). Plasma cholesterol and triglyceride were determined by using commercially available enzymatic kits (Biopacific Diagnostic Inc, BC, CA). Total free fatty acids (FFA) in plasma were measured by using NEFA-C Test Wako kit (Wako Pure Chemical Industries, VA).

Plasma lipoprotein fractions were separated by density gradient ultracentrifugation (Optima MAX Ultracentrifuge, Beckman coulter, CA, USA) at 100,000 rpm for 3, 4, and 6 hours to obtain the fraction of VLDL, LDL, and HDL, respectively, as described in elsewhere (Wasan *et al.*, 2001). The different triglyceride and cholesterol molecules were fractionated as follows: VLDL had a density (d) of 0.095-1.006 kg/L, LDL had d= 1.006-1.063 kg/L, HDL had d >1.063 kg/L (Wasan *et al.*, 2001). Triglyceride and cholesterol concentrations were determined for each of the fractions using enzymatic kits (Biopacific Diagnostic Inc, BC, CA).

Lipid content of the liver and adipose tissues was extracted by chloroform/ methanol (2:1, v/v) solution as described by Folch *et al.* (1957). Triglyceride and cholesterol concentrations were measured using commercially available enzymatic kit (Biopacific Diagnostic Inc, BC, CA).

5.2.2.5 Adipose tissue histology

Difference in epididymal histological was determined by analyzing two representative animals from each treatment group. Frozen epididymal adipose tissues were thawed and fixed in 10% (v/v) formalin. After 48 hours of fixation, tissues were dehydrated in graded alcohols, embedded in paraffin blocks, and cut into sections of 5 μm thick. Mounted sections were stained for light microscopy with haematoxylin and eosin using standard methods (Ludwig *et al.*, 2002). The histology analysis was performed by Dr. P. Nation, Health Sciences Laboratory Animal Services (HSLAS), University of Alberta.

5.2.3 Statistical analyses

Results were reported as the mean ± SEM. Statistical analysis was performed by using one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls (SNK) test as a post-hoc analysis to detect any significant differences among the groups. A p-value < 0.05 was considered statistical significant. Group means for body weight, plasma glucose and plasma insulin from OGTT were calculated by repeated measurement analysis. The differences of AUC between time points were determined using repeated measurement analysis and a SNK test was performed to identify differences. Group means of total AUC were performed by ANOVA, followed by SNK test as a post-hoc analysis.

5.3 Results

5.3.1 *Glucose Load Study*

5.3.1.1 Food intake and weight gain

During the acclimatization period the rats consumed 22.3 ± 0.3 g of food/d (n=10) and gained 1.2 ± 0.2 g/d.

5.3.1.2 Oral Glucose Tolerance Test

The initial plasma glucose did not differ between groups (Fig 5-3). The plasma glucose levels were higher at 15, 30, and 60 min in rats gavaged with the control (glucose solution without galactomannan) compared to those gavaged with the glucose solution containing either 0.5% or 1% (w/w) galactomannan ($p<0.01$). Plasma glucose levels were not significantly different among groups at 120 min. The peak plasma glucose level of control, 0.5% (w/w) galactomannan, and 1% (w/w) galactomannan groups was attained at 15 min, 30 min, and 60 min, respectively. Plasma glucose concentrations after the 1% galactomannan challenge were significantly lower than that of 0.5% (w/w) galactomannan challenge ($p<0.01$) at 15 and 30 min.

5.3.2 *Four-Week Feeding Study*

5.3.2.1 Food intake, body and tissue weights

Initial body weights did not differ among groups. Food intake was significantly lower in high GAL group at 16%, and 12% when compared with the control and low GAL groups, respectively ($p<0.05$, Table 5-2). Body weight of rats in all groups increased steadily with time. However, the average body weight of the rats fed the high

GAL group was significantly lower by 6% (on day 10) and by 11% (on day 28) compared to the control and the low GAL groups (Fig 5-4). Furthermore, the overall of body weight gain of rats in the high GAL group was significantly lower compared to the control and the low GAL groups ($p<0.05$, Table 5-2). There were no differences in overall body weight gain between the low GAL group and the controls. The ratio of weight gain to gram of food intake in all 3 groups was not significantly different (Table 5-2).

The low and high GAL groups had significantly less epididymal adipose tissue than the control group by 23% and 56%, respectively ($p<0.05$, Table 5-2). There were no significant differences in the weight of peri-renal adipose tissue depot among groups.

5.3.2.2 Oral Glucose Tolerance Test

Fasting plasma glucose in the high GAL group was significantly reduced compared to the low GAL and the control groups ($p<0.05$, Fig 5-5). After the OGTT, plasma glucose concentrations did not differ significantly at 15, 30, and 60 min. However, the plasma glucose concentration in the high GAL group was significantly lower at 120 min as compared to the low GAL and the control groups ($p <0.05$).

Area under the curve (AUC) between 0 to 120 min for glucose of high GAL group was significantly reduced compared to the control and low GAL groups (Table 5-3). Separate analysis of glucose AUC between 30 and 60 min confirmed that the AUC for the high GAL group was significantly lower than the control. Between 60 to 120 min, the AUC of both low and high GAL groups were significantly lower compared to the

control group. There were no differences in glucose AUC at 15 min (AUC 0 to 15 min), 30 min (AUC 15 to 30 min) among three groups.

Fasting plasma insulin in the high GAL group was significantly lower than that of the low GAL and the control groups ($p<0.001$, Fig 5-6). After the OGTT, plasma insulin of the high GAL group was significantly lower than the insulin levels of the low GAL and control groups at 15, 30, 60 and 120 min ($p<0.001$). There were no differences in plasma insulin concentrations between the control and the low GAL groups.

Feeding both low and high GAL diets to rats resulted in significantly lower AUC for insulin between 0 to 120 min compared to the control (Table 5-4). The AUC for the high GAL group was significantly lower than the control at all measured time points. In low GAL group, compared to the control group, there was a significantly lower AUC for insulin at every measured time point, except at 30 min (AUC 15 to 30 min).

5.3.2.3 Plasma total triglyceride, free fatty acids and total cholesterol concentrations

Both the low and high GAL groups had a significantly lower ($p<0.05$) plasma triglyceride concentrations, by 42% and 48%, respectively compared to the control (Fig 5-7). Plasma total cholesterol concentrations in both the low and high GAL groups were significantly lower than that of the control by 23% and 32%, respectively ($p<0.05$). Plasma FFA in the low GAL group was significantly lower by 15% than the control ($p<0.05$). There were no significant differences in the concentrations of plasma FFA between the high GAL group and the control.

5.3.2.4 Plasma lipoprotein triglyceride concentrations

VLDL triglyceride concentrations in all 3 groups were similar ($p>0.05$, Fig 5-8). High GAL group had 41% less LDL-TG concentration than the control group ($p<0.05$). Meanwhile, there were no statistical differences in LDL-TG concentrations between the low GAL and control groups. Concentrations of HDL-TG in the high GAL group were 50% lower than that of controls ($p<0.05$). There were no statistical differences of HDL-TG concentrations between the low GAL and control groups.

5.3.2.5 Plasma lipoprotein cholesterol concentrations

VLDL cholesterol concentrations in all 3 groups were similar ($p>0.05$, Fig 5-9). High GAL group had a lower LDL cholesterol concentration by 51% when compared to the control ($p<0.05$). There were no statistical differences of LDL cholesterol between the low GAL and control groups. HDL cholesterol concentration in the high GAL group was significantly lower (47%) than the control group ($p<0.05$). Concentration of HDL cholesterol was not statistically different between the low GAL and control groups.

5.3.2.6 Hepatic triglyceride and cholesterol concentrations

The low GAL group had 45% lower concentrations of hepatic triglyceride ($p<0.05$) than the control group (Fig 5-10). There were no statistical differences in hepatic triglyceride concentrations between the high GAL and control groups. Hepatic cholesterol concentration was lower in low and high GAL groups, 34% and 35%, respectively, as compared to control rats ($p<0.05$).

5.3.2.7 Adipose triglyceride concentrations

Epididymal triglyceride concentrations in the high GAL group were reduced by 20% when compared to the control group ($p<0.05$, Fig 5-11). There were no statistical differences in adipose tissue triglyceride concentrations between the low GAL and control groups.

5.3.2.8 Adipose tissue histology

The histology of the epididymal adipose tissue of the high GAL group was visibly different from the control and low GAL groups (Fig 5-12). Tissue from the control and low GAL groups appeared similar appearance with fat cells dispersed throughout the tissues. However in the high GAL group, fat cells appeared to cluster throughout the tissues. These clusters appeared to be comprised of large fat cells surrounded by small fat cells. The capillaries in the high GAL group were filled with erythrocytes.

5.4 Discussion

5.4.1 *Glucose Load Study*

Results of the glucose load study demonstrated that combining 1% (w/w) galactomannan in a glucose bolus, compared to a 0.5% (w/w) galactomannan solution reduced blood glucose levels at 15 and 30 min post OGTT (Fig 5-3). In agreement with other studies (Edward, 1990; Ehrlein and Stockmann, 1998), our results indicated that feeding galactomannan reduced the rise in plasma glucose after an OGTT. This reduction in blood glucose level has been suggested to be due to the fiber increasing the viscosity of the contents of the stomach and small intestine, and increasing the thickness of the UWL

which would inhibit the transport of glucose to the mucosal surface (Johnson and Gee 1981; Flourie *et al.*, 1984). Another possible mechanism to lower plasma glucose may relate to the rate of gastric emptying. Earlier studies had shown that viscous fiber delayed the duration of gastric emptying, thereby slowing the release of nutrients into the small intestine, resulted in a lower blood glucose concentration Torsdottir *et al.*, 1989; Macdonald, 1996; Schneeman, 2002). In the present study, the difference in plasma glucose concentrations between 0.5% (w/w) and 1% (w/w) galactomannan-containing solutions, is suggestive of an effect on intestinal viscosity. This is supported by the result shown in chapter 3, where we demonstrated that the viscosity of 1% (w/w) galactomannan solution was 10 fold higher than that of 0.5% (w/w) galactomannan solution at 37°C (Table 3-1).

5.4.2 *Four-Week Feeding Study*

5.4.2.1 Food intake and weight gain

Rats fed with the high GAL diet had significantly lower food intake and weight gain when compared to rats fed with the low GAL or the control diets ($p<0.05$, Table 5-2). However, the ratio of body weight gain to food intake in all 3 groups was similar. These data indicated that the reduction in weight gain in the high GAL group was likely due to reduced food intake, rather than malabsorption. There are several possible mechanisms to explain the reduced intake in the high GAL group. A possible mechanism that could be hypothesized might be due to the lower rise in plasma insulin concentration when fed the high GAL diet (Fig 5-6). Anderson (1990) reported that a decrease in plasma insulin concentration can decrease in food intake because insulin stimulates

appetite. Another possible mechanism may be due to slowing down of gastric emptying. It was shown that the increased viscosity of the gastric content produced by the hydrophilic character of guar gum slows the gastric emptying rate, increases the satiety and consequently decreases food intake (Krotkiewski, 1984; Kovacs *et al.*, 2001). The effect of high viscous polysaccharides on gastric distension may act to suppress postprandial food intake (French and Read, 1994). There is also evidence to suggest that dietary fibers influence the secretion of intestinal hormones [Cholecystokinin (CCK) and glucagon-like-peptide-1 (GLP-1)]. Both of these hormones have been found to signal the satiety center in the brain and thereby reducing appetite (Haber *et al.*,1977; Lavin *et al.*,1998).

5.4.2.2 Glucose Homeostasis

After feeding the test diets for 3 weeks, fasting plasma glucose was least in the high GAL group (Fig 5-5). In the high GAL group, the reduction in fasting plasma glucose was parallel with the significant reduction of fasting plasma insulin. Since insulin regulates hepatic glucose production (Cherrington *et al.*, 1998) the reduced fasting plasma insulin may have contributed to the lower level of fasting plasma glucose. Another possible mechanism to explain the result of lower fasting glucose in high GAL group is that fermentation of fibers by colonic bacteria produces more propionate; the latter inhibits glucose production in hepatocytes, thus improving glucose level (Anderson and Bridges, 1984; McCarty, 2002).

There were no statistical differences in plasma glucose levels at 15, 30 and 60 min post OGTT in all groups (Fig 5-5). However, the AUC of glucose at 60 min and 120 min

of both low and high GAL groups were significantly lower than the control (Table 5-3). The insulin peak after OGTT of high GAL group was significantly lower at every measured time point compared to the control. Although the lower insulin peak after OGTT was not found in low GAL group, the AUC of insulin in the low GAL group was significantly reduced at 15, 60, and 120 min compared to the control. The increase in plasma insulin concentration reflects an increased rate of insulin released into the blood in response to glucose absorption and hyperglycemia (Murao *et al.*, 1994). The subsequent decline probably represents a moderation in the rate of insulin release and an increased rate of insulin removal from blood (William *et al.*, 2001). In the present study, the difference in total-insulin AUC between the low GAL group and control was 17%, and 42% between the high GAL group and control (Table 5-4). These are higher than corresponding differences in total-glucose AUC at 4% between the low GAL group and control, and 9% between the high GAL group and control (Table 5-3). These findings suggest that feeding rats with galactomannan-containing diet for 3 weeks may improve insulin sensitivity, especially in high GAL group. However, this needs to be further elucidated by using insulin clamp technique. Our finding is in agreement with other investigators who found similar effects of soluble fiber on improving insulin sensitivity (Fung *et al.*, 2001; Wirfalt *et al.*, 2001).

5.4.2.3 Size of adipose tissue

Feeding both low and high GAL diets resulted in significantly lower the weight of epididymal adipose tissue compared to the control group (Table 5-2). There were, however, no statistical differences in peri-renal adipose tissue among groups. The high

GAL diet was more effective than low GAL and control diets in reducing fat mass of epididymal adipose depot (Table 5-2). This was consistent with the lower epididymal triglyceride level in high GAL group (Fig 5-11). Furthermore, the small fat cells, the increased amount of capillaries (Fig 5-12), and the unaltered plasma FFA (Fig 5-7) suggest that the metabolic activity of this adipose tissue depot is augmented in the high GAL group. We speculate that the low intake in high GAL group may contribute to the stimulated lipolysis of the fat cells. Since adipose tissue deposition is the result of the difference between energy intake and energy expenditure (Soria *et al.*, 2002) the less deposition would be expected in the low intake of growing rats (4 week old). It has also been shown that the turnover of lipogenesis and lipolysis is greater in visceral than in subcutaneous fat, probably as a consequence of the greater deposition of fat in visceral tissue (Jenkin *et al.*, 2000). It could be possible that epididymal is more dynamic than peri-renal adipose tissue. However, since food intake was reduced in this group to confirm this hypothesis one will have to include a pair-fed group in future experiments.

5.4.2.4 Plasma triglyceride levels

Feeding both low and high GAL diets resulted in significantly lower plasma total TG compared to the controls (Fig 5-7). The reduction in total TG was due to a decline in both LDL- and HDL-TG concentrations (Fig 5-8). Concentrations of VLDL-TG were not altered in either the low or high GAL groups as compared to controls. Two factors that determined serum levels of VLDL-TG are the rate of hepatic secretion of VLDL-TG and the capacity for hydrolyzing TG into LDL in the circulation (Munilla and Herrera, 1997). In this study, the production of VLDL-TG in the high GAL group is most likely

due to the augmented influx of FFA into the liver. However, this is not the scenario in the low GAL group because the concentration of FFA was reduced while the VLDL-TG concentrations were similar among the groups. The similar VLDL-TG of low GAL and high GAL groups compared to the control might be explained by the increased TG production in the liver. It has been shown that decreased enterohepatic circulation of bile acids increases the activity of phosphatidic acid phosphatase, a rate-limiting enzyme responsible for the conversion of α-glycerol phosphate to TG (Angelin *et al.*, 1986; Ast and Frishman, 1990). The increased activity of this enzyme was reported to be associated with an increase in the TG content of VLDL leading to increased size of VLDL (Shepherd, 1989).

5.4.2.5 Hepatic Triglyceride

Low GAL diets fed to rats for 4 weeks significantly reduced the hepatic triglyceride compared to the control (Fig 5-10). Our result is in agreement with other investigators (Artiss *et al.*, 2006; Beylot, 2005). The mechanism most likely associated with this hepatic-triglyceride depletion may be related to the capacity of soluble fiber to delay sucrose absorption in the intestinal lumen (Ebihara *et al.*, 1989). However, the lower hepatic triglyceride was not seen in high GAL groups. Increased hepatic triglyceride synthesis in high GAL group may have contributed to the increased FFA (Fig 5-7 and 5-10). Despite the increased hepatic triglyceride concentrations in the high GAL and control groups, the levels were still within normal range when compared to other reports (Oda *et al.*, 1994; Choi *et al.*, 1998).

5.4.2.6 Plasma cholesterol levels

Feeding galactomannan significantly lowered plasma total cholesterol by 23%-32% when compared to the controls (Fig 5-7). The reduction was due to a decline in both LDL and HDL cholesterol (Fig 5-9). VLDL cholesterol was not altered in the low or high GAL groups, which agrees with Chen, *et al.* (1981). Chen *et al.* (1981) found that feeding dietary fiber selectively lowered serum LDL cholesterol but did not alter VLDL-, or HDL- cholesterol concentrations. The total cholesterol lowering effects of galactomannan observed in our study support previous assertions that increasing the viscosity of luminal contents by soluble fiber augments the total cholesterol lowering effects of a fiber (Davidson *et al.*, 1998; Anderson *et al.*,1983;1986;1988). Previous studies with guar gum have suggested a direct effect of guar on lower serum cholesterol by binding the bile acids in the intestinal lumen (Hansen *et al.*, 1983; Turner *et al.*, 1990) or reducing ileal bile acid absorption through impeding bulk diffusion to the luminal surface (Kritchevsky and Story, 1974), and increasing fecal bile acid loss (Everson *et al.*, 1992; Jenkins *et al.*, 1993; Marlett *et al.*, 1994; Seal and Mathers, 2001). As a consequence, an increased hepatic conversion of cholesterol to bile acids occurs, thus lowering cholesterol in the circulation (Turner *et al.*, 1990).

5.4.2.7 LDL cholesterol

Feeding rats with 5% (w/w) galactomannan significantly reduced plasma LDL cholesterol compared to the control (Fig 5-9). The possible mechanism is that viscous fibers bind bile acids in the small intestine leading to increased fecal bile acid excretion (Lia *et al.*, 1995). A decrease in hepatic concentrations of bile acids would stimulate the

activity of cholesterol 7 α-hydroxylase, the rate-limiting enzyme in bile acid synthesis. Lowering bile acid pool was also reported to increase the activity of HMG-CoA reductase, the rate limiting enzyme in the hepatic cholesterol synthesis pathway (Favier *et al.*, 1998; McCarty, 2002). Theoretically, an increased activity of HMG-CoA reductase would increase cholesterol synthesis (review in Schmitz and Langmann, 2006), however there was no rise in plasma cholesterol in the present study. This might be explained by a shunting of the newly formed cholesterol into the bile acid synthesis pathway (Ast and Frishman, 1990). It had been suggested that the increase synthesis of cholesterol might cause the hepatocyte cell surface receptors for LDL particles to be altered either quantitatively by increasing in number, or qualitatively by increasing in affinity for the LDL particle (Fernandez, 1995; Brown *et al.*, 1999). In the present study, the significant reduction of LDL cholesterol in the high GAL group (Fig 5-9) may be indicating an increased clearance of LDL cholesterol. Although the LDL cholesterol in low GAL group (28% reduction compared to the control) was not significantly different from the high GAL group (51% reduction compared to the control), the trend towards a concentration effect is interesting. This trend might be due to the difference in viscosities induced by the two diets. In our previous work (chapter 3), the viscosity of galactomannan was increased 10-fold when the concentration of galactomannan increased 2-fold (Table 3-1).

5.4.2.8 HDL cholesterol

A meta-analysis concluded that soluble fibers (i.e., guar gum and psyllium) reported that consuming these significantly reduced HDL cholesterol when they were

consumed in the high dose range (12-30 g/d) (Brown *et al.*, 1999). In the present study, the HDL cholesterol in high GAL group was significantly lower (47% reduction) than the control (Fig 5-9). In rats, circulating levels of cholesterol are generally lower than in humans (Ferezou and Chevallier, 1986), and 60% of cholesterol is transported by HDL (Fidge and Poulis, 1978). LDL carry about 60% of plasma cholesterol in healthy subjects (Carlson and Erricsson, 1975; Avramoglu *et al.*, 2003). It is most likely that the lower HDL cholesterol in the present study may due to the increased demand of cholesterol for bile acid synthesis as mentioned in section 5.4.2.7. However, this needs to be further tested using other obese or hypercholesterolemic animal models i.e., Zucker diabetic fatty (ZDF *fa/fa*) rats, or cholesterol-fed Guinea pigs, to confirm an effect prior to conducting human trials.

Zucker obese (*fa/fa*) and ZDF (*fa/fa*) rats, inherit obesity, have a marked increase in plasma lipids and lipoproteins in particular VLDL (Schonfeld *et al.*, 1974; Sparks *et al.*, 2000; Blay *et al.*, 2001; Tovar *et al.*, 2005). It has been shown that hepatic overproduction of lipoproteins contributed to the development of hyperlipidemia in these animals (Witztum and Schonnfeld, 1979; Azain *et al.*, 1985). A review of animal model to study lipoprotein metabolism (Fernadez and Volek, 2006) has been shown that Guinea pigs carry the majority of the cholesterol in LDL which is similar to the human situation. Fernandez *et al.* (1992) had shown that dietary fat saturation (15% w/w, 35% calories) increased guinea pig plasma LDL levels by altering LDL receptor-mediated catabolism.

In humans, the LDL:HDL cholesterol ratio is a risk factor for CVD (Ascherio, 2006). It is found that an increase in the ratio of LDL to HDL by one unit may contribute to a 75% increase in the risk of myocardial infarction (Austin, 1989). Although there was

adverse effect on lowering HDL cholesterol in the high GAL group, no significant difference of LDL:HDL ratio was found between control and high GAL groups. The LDL:HDL ratio was: 0.61 in the control, 0.57 in the low and high GAL groups.

5.4.2.9 Hepatic cholesterol

Feeding both low and high GAL diets resulted in significantly lower hepatic cholesterol content compared to the control (Fig 5-10). Consistent with other studies, viscous fibers have been shown to reduce liver cholesterol concentration (Wilson *et al.*, 2004; Nicolle *et al.*, 2004). The reduction of hepatic cholesterol level in this study may have influenced the conversion of cholesterol to bile acids as described in section 5.4.2.7. Another possible mechanism is that galactomannan fermented in the colon by microflora produces proprionate. Increased colonic productin of proprionate has been demonstrated to inhibit HMG-CoA reductase activity, resulting in lower hepatic cholesterol biosynthesis (Jenkins *et al.*, 2000).

In summary, galactomannan intake appears to have a promising glycemic-preventive effect in the postprandial states since it delayed the absorption of glucose leading to lowered plasma glucose during the OGTT procedures (i.e. glucose load study). In the four-week feeding experiment, galactomannan at 2.5% and 5% (w/w) reduced plasma triglyceride, cholesterol and hepatic cholesterol concentrations. Galactomannan at 5% (w/w) resulted in lower food intake, which may have enhanced the release of plasma FFA, and in turn, lead to the least amount (i.e. weight) of epididymal adipose

tissue. However, it is too early to recommend galactomannan as a means for treatment, as more studies in obese animal models must be performed.

Table 5-1. Composition of Diets

	Control	low GAL	high GAL
Ingredients (g/100g of diet):			
Casein	20.0	20.0	20.0
Sucrose	52.0	52.0	52.0
Corn oil	5.0	5.0	5.0
Stearin	7.4	7.4	7.4
Linseed oil	0.5	0.5	0.5
DL-methionine	0.3	0.3	0.3
Choline bitartrate	0.2	0.2	0.2
Vitamin mix (AIN-93-VX)	1.1	1.1	1.1
Mineral mix (AIN-93-M)	3.5	3.5	3.5
Cellulose	10.0	7.5	5.0
Galactomannan	0.0	2.5	5.0

The composition of diets was modified from AIN 93G (Reeves *et al.*, 1993). Polyunsaturated fatty acid to saturated fatty acid (P/S) ratio was 0.4 based on approximate North American fat intake (Hayes, 2002).

Table 5-2.Effect of galactomannan on food intake, weight gain, and adipose tissue weight

	Control	low GAL	high GAL
Food intake (g/d)	26.8±0.8[a]	25.6±0.8[a]	22.6±0.8[b]
Body weight gain (g)	165.8±9.7[a]	157.4±10.3[a]	124.2±10.3[b]
Body weight gain/ intake ratio	6.26±0.1	6.10±0.1	5.48±0.1
Adipose tissue			
- epididymal adipose tissue (g)	5.90±0.3[a]	4.57±0.3[b]	2.58±0.3[c]
% of body weight	1.5%	1.2%	0.7%
- peri-renal adipose tissue (g)	1.38±0.1	1.23±0.1	0.91±0.1
% of body weight	0.3%	0.3%	0.26%

Values represent means ± SEM of control (n=9), low GAL (n=8), high GAL (n=8).
[a-c] values in a row not sharing a common superscript differ significantly at $p<0.05$.

Table 5-3. Area under the curve of plasma glucose after OGTT

Treatment	15 min	30 min	60 min	120 min	Total AUC (0 to120 min)
Control	144.5±1.8	196.5±1.8	386.7±4.3 [a]	633.8±5.8 [a]	1361.5±12.5 [a]
Low GAL	149.7±1.8	199.3±1.8	364.4±4.3 [b]	597.4±5.8 [b]	1310.9±12.5 [a]
High GAL	144.5±1.8	191.3±1.8	348.1±4.3 [b]	559.8±5.8 [c]	1239.9±12.5 [b]

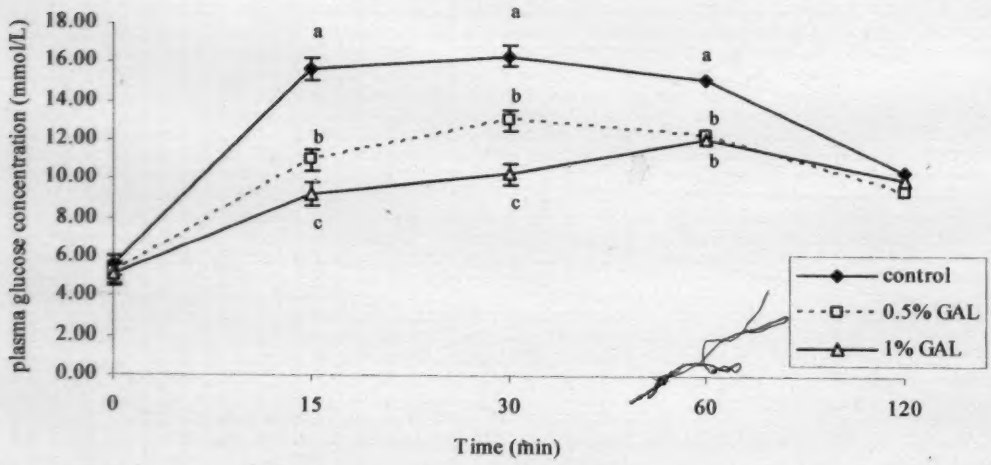
[a-c] Different letters following means±SEM within a column indicate a significant difference at the 0.05 level.

Table 5-4. Area under the curve of plasma insulin after OGTT

Treatment	15 min	30 min	60 min	120 min	Total AUC (0 to 120 min)
Control	1.45±0.01 [a]	1.87±0.05 [a]	3.49±0.09 [a]	5.2±0.1 [a]	12.1±0.3 [a]
Low GAL	1.28±0.01 [b]	1.81±0.05 [a]	3.04±0.09 [b]	3.9±0.1 [b]	10.1±0.3 [b]
High GAL	0.73±0.01 [c]	1.20±0.05 [b]	2.37±0.09 [b]	2.8±0.1 [c]	7.1±0.3 [c]

[a-c] Different letters following means±SEM within a column indicate a significant difference at the 0.05 level.

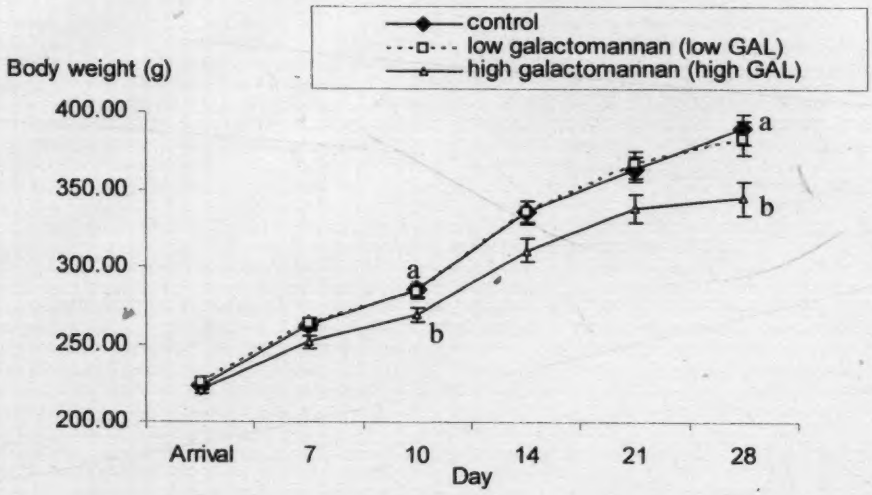
Fig 5-3. Plasma glucose response to an OGTT in the glucose load study



Values represent means± SEM in 10 rats.

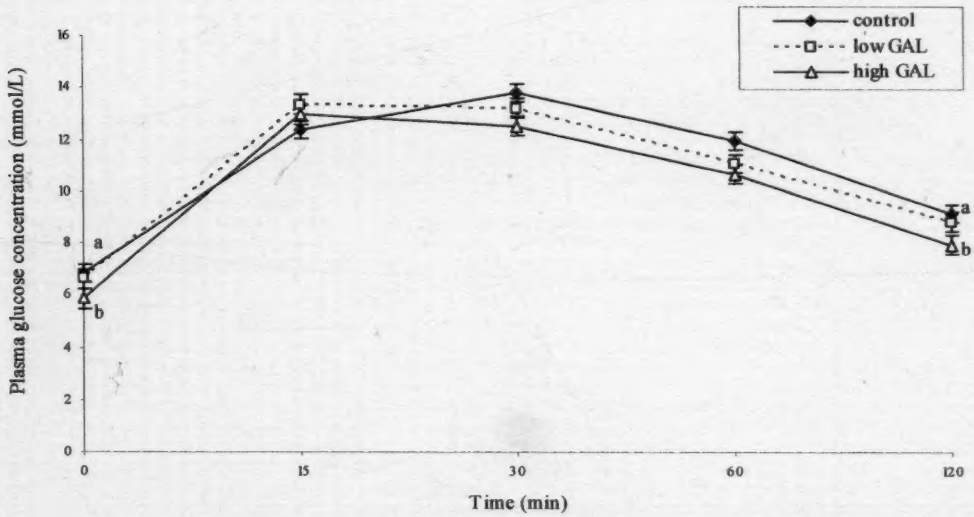
[a-c]Values in the same time point not sharing the same superscript are significantly different ($p < 0.01$).

Fig 5-4. Body weight of rats fed with different diets for 4 weeks



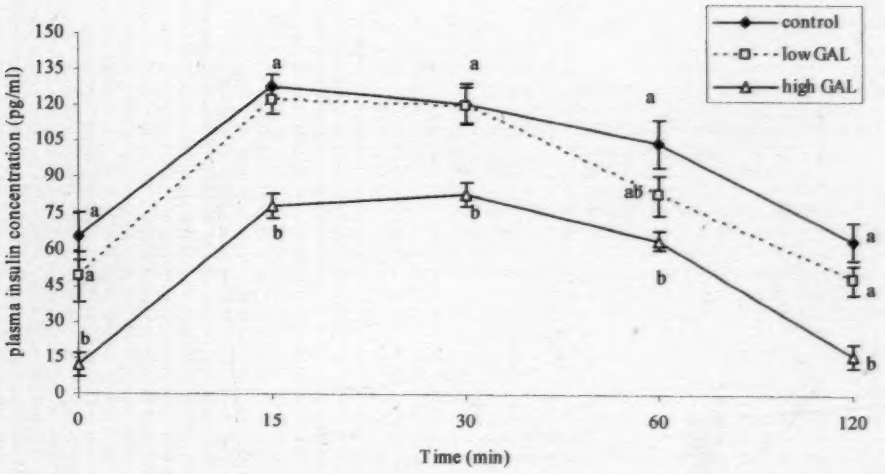
Values represent means ± SEM of control (n=9), low GAL (n=8), high GAL (n=8).
[a,b] Values in the same time point not sharing the same superscript are significantly different ($p < 0.05$).

Fig 5-5. Plasma glucose response to the OGTT in rats fed one of three diets for 3 weeks



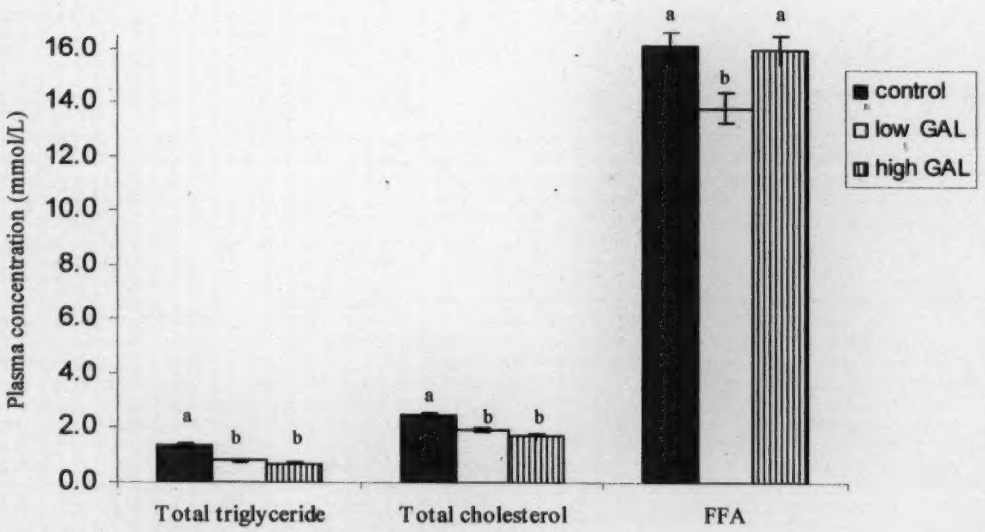
Values represent means ± SEM of control (n=9), low GAL (n=8), high GAL (n=8).
[a,b]Values in the same time point not sharing the same superscript are significantly different ($p < 0.05$).

Fig 5-6. Plasma insulin response to the OGTT in rats fed one of three diets for 3 weeks



Values represent means± SEM of control (n=9), low GAL (n=8), high GAL (n=8).
[a-c]Values in the same time point not sharing the same superscript are significantly different ($p < 0.05$).

Fig 5-7. Plasma levels of total-triglyceride, total cholesterol, and FFA in rats fed with different diets for 4 weeks

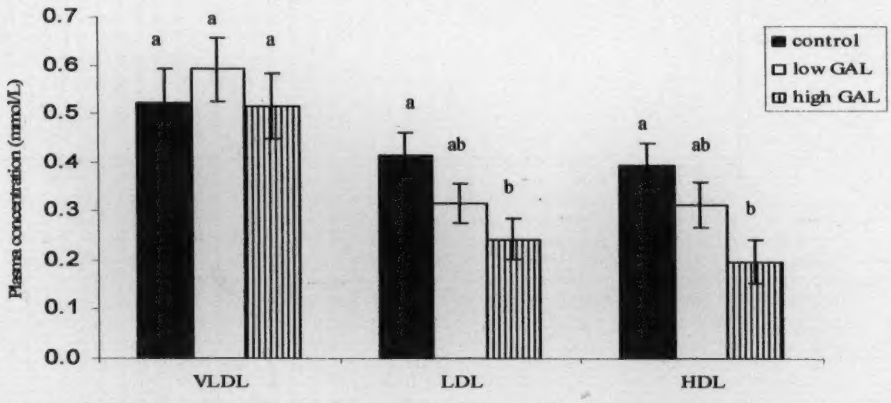


Values represent means ± SEM of control (n=9), low GAL (n=8), high GAL (n=8).

[a,b]different superscripts indicate significant difference at $p<0.05$ among control, low GAL and high GAL groups.

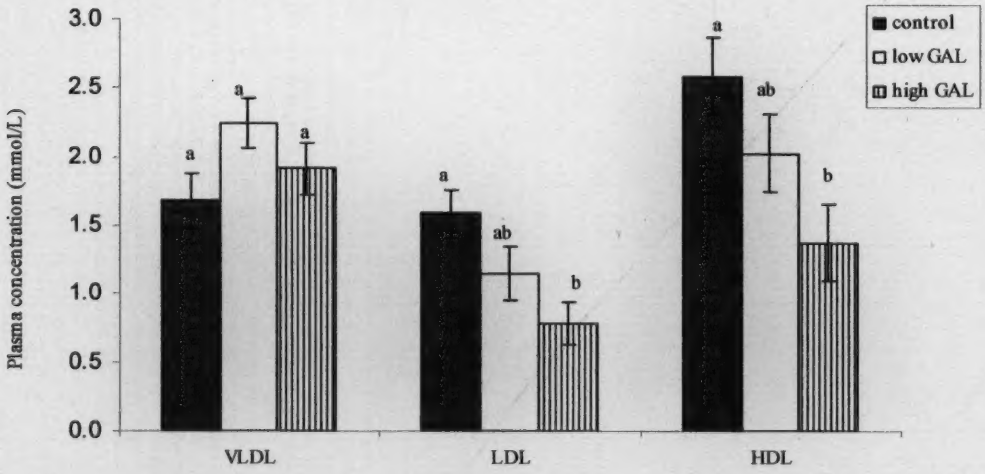
To convert value for triglyceride to mg/dl, divide by 0.01129;
To convert value for cholesterol to mg/dl, divide by 0.02586;
To convert value for free fatty acid to mg/dl, divide by 0.0355 (Kasim-Karakas *et al.*, 2000)

Fig 5-8. Lipoprotein triglyceride levels in rats fed with different diets for 4 weeks



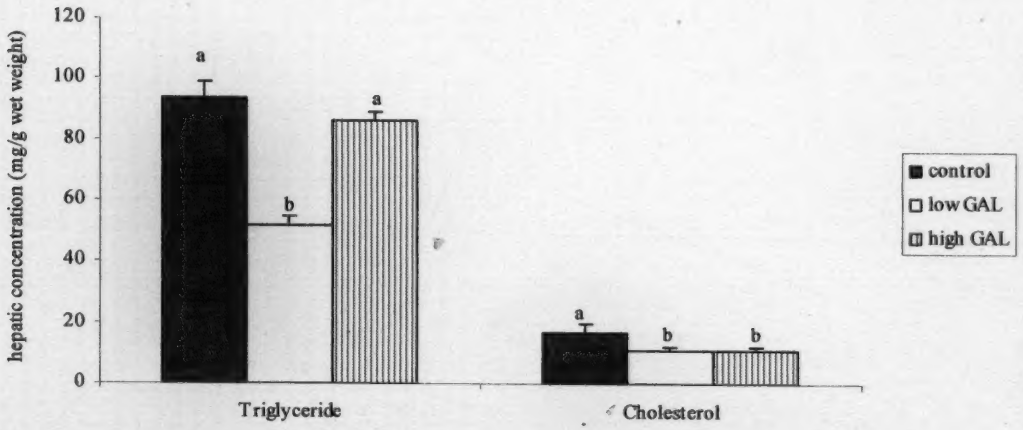
Values represent means ± SEM of control (n=9), low GAL (n=8), high GAL (n=8). [a,b]different superscripts indicate significant difference at $p<0.05$ among control, low GAL and high GAL groups.

Fig 5-9. Lipoprotein cholesterol levels in rats fed with different diets for 4 weeks



Values represent means ± SEM of control (n=9), low GAL (n=8), high GAL (n=8). [a,b]different superscripts indicate significant difference at $p<0.05$ among control, low GAL and high GAL groups.

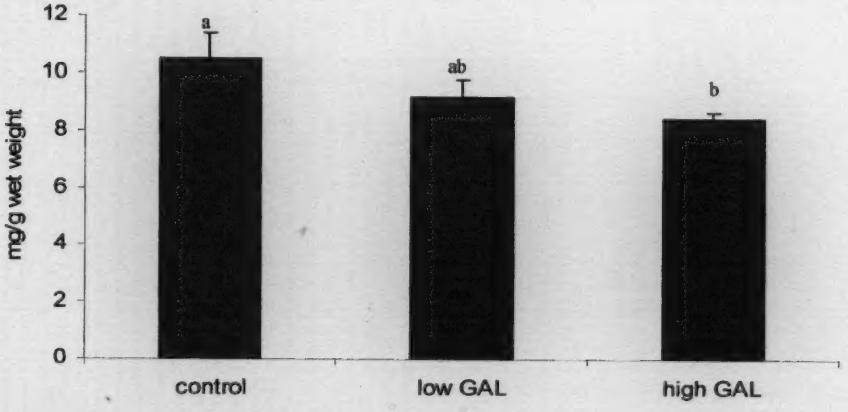
Fig 5-10. Hepatic triglyceride and cholesterol levels in rats fed with different diets for 4 weeks



Values represent means ± SEM of control (n=9), low GAL (n=8), high GAL (n=8).

[a,b]different superscripts indicate significant difference at $p<0.05$ among control, low GAL and high GAL groups.

Fig 5-11. Epididymal triglyceride level in rats fed with different diets for 4 weeks

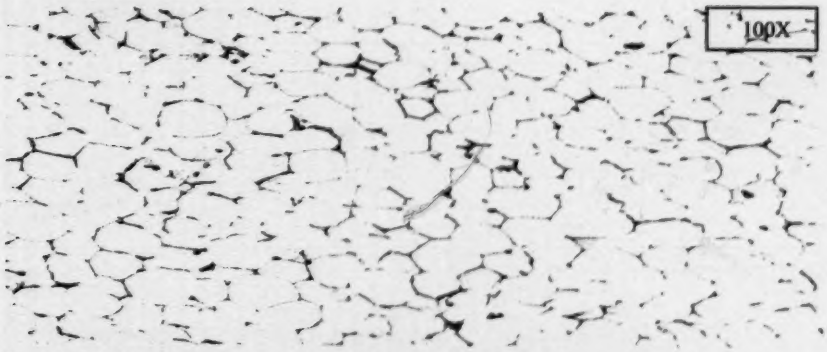


Values represent means± SEM of control (n=9), low GAL (n=8), high GAL (n=8).

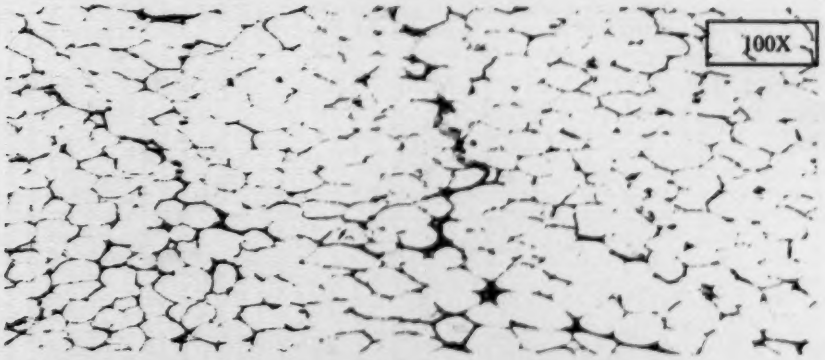
[a,b]different superscripts indicate significant difference at $p<0.05$ among control, low GAL and high GAL groups.

Figure 5-12. Histology of epididymal adipose tissues from rats fed with different diets for 4 weeks

a) Control group



b) Low GAL group (2.5% galactomannan containing diet)



c) High GAL group (5% galactomannan containing diet)

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Chapter 6

General summary and discussion

6.1 Summary of results

The first objective of this research was to test the following hypotheses:

I. The chemical compositions of Alberta-grown fenugreek seeds were not different from that of Indian fenugreek seeds.

This hypothesis was supported by the results reported in chapter 2. Four new Alberta-grown lines of fenugreek seed and Indian seed had similar contents of most of the chemical constituents. However, the galactomannan content of the four Alberta-grown lines were in the range of 33-37% (w/w) compared to the Indian line (23%, w/w).

II The fractionated-extraction method gave yield and purity of galactomannan greater than the chemical-extraction method.

The results presented in chapter 2 supported this hypothesis. Of the two different extraction methods investigated, extraction involving fraction A (seed coat/ aleurone/ endosperm) was simple and afforded the highest recovery of galactomannan (89% w/w) with 16% yield of original seeds. The product was white in color with 91.4% (w/w) purity. The use of fraction A to extract galactomannan limited the need for chemical treatments.

III Viscosity and viscoelastic properties of galactomannan were not affected by adding 10% or 20% (w/w) sucrose.

The experiments described in chapter 3 provided evidence for this hypothesis. Viscosity of galactomannan extract increased non-linearly when the concentration was increased from 0.25% (w/w) to 1% (w/w) at 0.25% (w/w) increments. Increased temperature decreased the viscosity of solutions. Adding sucrose at 10% or 20% (w/w) did not affect the viscosity nor elastic properties of galactomannan extract up to 0.75%, and 1% (w/w) concentration. Galactomannan showed pseudoplastic behaviour which is suitable for a wide range of food and non-food applications.

The second objective of this research was to test the following hypotheses:

IV Galactomannan extract inhibited glucose uptake *in vitro* in intestine of both lean and obese JCR rats.

This hypothesis was supported by the results reported in chapter 4. Two factors were identified that influenced the rate of glucose uptake: galactomannan and glucose concentration. In the presence of 2 mM glucose, galactomannan (ranged 0.25-0.5%, w/w) significantly reduced the rate of glucose uptake by 55-73% in the jejunum and by 63-64% in the ileum as compared to the control. In the presence of 32 mM glucose, galactomannan at 0.1-0.35% (w/w) concentration significantly reduced the rate of glucose uptake by 26-33% in the jejunum and by 22-35% in the ileum as compared to the control.

V Feeding galactomannan solution together with glucose solution reduced the plasma glucose level of rats.

The results reported in chapter 5 support this hypothesis. Both 0.5% and 1% (w/w) galactomannan solution mixed with 75% w/w glucose solution (3 g/KgBW) provided to rats by gavage resulted in a significantly lowered plasma glucose concentrations compared to a placebo solution without galactomannan. At 15 and 30 min after OGTT, 1% (w/w) galactomannan fed rats had significantly lowered plasma glucose by 16% and 21%, respectively compared to the 0.5% (w/w) galactomannan solution.

VI Feeding a diet with galactomannan reduced plasma glucose, insulin, cholesterol, and TG concentrations, and adipose weight of rats.

This hypothesis was supported by the results reported in chapter 5. Rats fed with 5% (w/w) galactomannan in a high-sucrose diet had significantly lowered fasting plasma glucose by 11-15% and plasma insulin by 75-81% compared to 2.5% (w/w) galactomannan and control groups. Feeding both 2.5% and 5% (w/w) galactomannan diets resulted in significantly lowered plasma total triglyceride by 42-48%, total cholesterol by 23-32%, LDL-cholesterol by 28-51%, and epididymal adipose weight by 23-56% when compared to control. However, HDL-cholesterol was significantly lowered by 47% in rats fed with 5% galactomannan when compared to the control. Rats fed with 5% (w/w) galactomannan had significantly lower food intake by 12-16%, and body weight gain by 21-25% compared to other groups.

6.2 General Discussion

Fenugreek is largely cultivated in India, Pakistan, and China (Ayers, 2000). It has been used as spice and forage crop. India produces about 35,000 tons of fenugreek annually and is one of the major producers and exporters of fenugreek in the world (Ayers, 2000). Fenugreeks are currently developed as an annual forage crop at Lethbridge Research Center (Lethbridge), Alberta because they fix nitrogen, improve soil for subsequent crops and have high crude protein content and digestibility for animal feed (Rochon *et al.*, 2004). The relevance to Alberta agriculture is that fenugreek has the potential to be a new annual forage crop. In comparison to conventional alfalfa, fenugreek yielded as much dry matter with approximately the same nutritional quality forage and did not induce bloat in cattle (Goonewardne, 1996). The identification of this new alternate forage crop as cattle feed and for export as dehydrated product could result in economic gain for Alberta agriculture. The known glucose and lipid-lowering effects of the Asian grown seed (review in Srinivasan, 2006) suggest that the Canadian grown fenugreek may also have beneficial health effects. This has the potential to further enhance the economic value of this new crop in Western Canada. This study investigates its seed benefits in both food application and health benefit.

Seeds of four fenugreek new varieties, namely Amber, F-70, F-86, L-3314, grown in Alberta and seeds of a variety grown in India had similar anatomical features as assessed by SEM (Appendix 5) and lipid content (chapter 2). However, the four varieties grown in Canada had higher crude protein (29%-32 %, w/w) and galactomannann (33%-37%, w/w) contents as compared to the Indian variety seeds, 26% and 23% (w/w), respectively. In agreement with other reports (Reid, 1985; Sharma, 1986; Ullmann,

1987; Mansour and El-Adawy, 1994; Brummer *et al.*, 2003), our results suggest that geographical location and conditions of cultivation play a role on the seed compositions.

Fenugreek seeds have not been widely consumed although it has potential health benefit. One reason could be its bitterness and pungent maple odor, due to the content of oil, steroidal saponins, and alkaloids. For a 100 g of mature seeds, there are approximately 7.5 g lipids, 2g sapogenins, 2 g alkaloids (Ravindran, 1997). Our preliminary work for fenugreek product development showed that adding 15 g powder of defatted-fenugreek seeds in a 762 g loaf had a moderate level of sensory acceptability with bitter aftertaste detected (Appendix 13). It was difficult to reduce the bitter taste when fenugreek powder was added to products with low moisture contents such as cookies and snack bars. We used other products that have high moisture, such as loaf. The result of this work suggested that although using loaf to improve the moisture and cocoa powder to mask the bitter taste, defatted fenugreek powder can only be added to about 1.96% (w/w), without significantly affecting consumer acceptability.

Another reason that fenugreek seeds have not been widely used is the limited number of studies that suggest it confers additional health benefits. Pervious studies were performed using whole or defatted fenugreek seeds at doses ranging from 5-100 g/day in either healthy, T2D, hypercholesterolemic subjects (Sharma and Raghuram, 1990; Raghuram *et al.*, 1994; Gupta *et al.*, 2001). This high dose amount would not be possible to consume as a daily dietary supplement form or as a mix-ingredient foods. To overcome this problem, one needs: 1) the development of an extraction method to isolate the active components from fenugreek seeds and 2) to define its effect on health indices involved in chronic diseases, such as blood glucose and lipids. Although there are a

number of potentially bioactive components in fenugreek we selected galactomannan because it is a soluble fiber, which has potential to lower plasma glucose and plasma lipid (review in Delzenne, 2002). North Americans have fiber intake lower than the recommendation, which suggest an intake of 14 g of dietary fiber/ 1,000 kcal, particularly from cereals, to promote heart health (Institute of medicine, 2005). Galactomannan extracted from fenugreek could provide an alternative fiber source for consumers to meet their fiber recommendations.

Fenugreek-galactomannan extraction is less studied. The existing method is time consuming and gives low yield, thus is of low cost-benefit to the producer. Moreover, the extract has high protein contamination. If fenugreek is to provide a valuable fiber source for the human diet, it is essential to develop an extraction method that provides high purity and yield of galactomannan. The first extraction method developed in this research provided high yield and purity of galactomannan. However, this method was time consuming. It took 5 days from the beginning of the procedure to obtain the final product. It was important to develop a new method to compare with the first one. We developed a novel method which was simple and no chemical reagents used in the procedure (chapter 2). The second method took 2 days from the beginning to obtain the final product.

Galactomannan obtained in this research was tested for properties of viscosity and viscoelastic in various temperatures, including the presence or absence of sucrose in the test solutions (chapter 3). It was shown that there were changes in the rheological and thermal properties of gums when sugar or salt was added in the solution (Papageorgiou and Kasapis, 1995). LBG-galactomannan at 1% (w/w) concentration resulted in a

decrease in the strength of cryogels with increasing concentrations of added sucrose (40-60%, w/w) (Doyle *et al.*, 2006). Our results showed that viscosity and viscoelastic properties of galactomannan extract were not affected by the added sucrose. In addition, the flow behavior index and the consistency coefficient of fenugreek-galactomannan dispersions were not affected by the added sucrose, and all dispersions at varying concentrations confer pseudoplastic liquid properties. These properties coupled with non-thixotropic nature make the fenugreek-galactomannan suitable for a wide range of food and non-food applications.

The combined results of *in vitro* and *in vivo* experiment in this research support that galactomannan-containing diets delayed absorption of nutrients, specifically glucose, fatty acids, and cholesterol. Figure 6-1 is a schematic representation of possible mechanisms by which galactomannan could reduce plasma glucose and lipid.

It has been shown that viscous polysaccharides inhibit glucose uptake by increasing the apparent thickness of the UWL resistance in the bulk phase. This has been determined by measuring the diffusion of the probe molecule to the mucosal surface (Jonhson and Gee 1981, Elsenhans *et al.*, 1984). The present study confirmed that increasing viscosity (by increasing the concentration of galactomannan ranged 0.1-0.5% w/w in the tested solutions) was associated with reduction in the rate of glucose uptake in the small intestine of JCR rats by 22-73% compared to the control (chapter 4). However, unexpectedly the high galactomannan concentration (0.5%, w/w) mixed with 32 mM glucose did not result in a significant reduction in the rate of glucose uptake compared to the control. Consistent with our results, it has been reported that mixing a large dose of guar gum (0.5%,w/w) in combination with a high glucose concentration (28 mM) had no

additional effect on lowering glucose uptake in the small intestine *in vitro* of rats as compared to a small dose of guar gum (0.1-0.25%, w/w) (Johnson and Gee,1981). Our results suggest that 32 mM glucose molecule might have high affinity to water molecule in the solution, causing a reduction of viscosity of galactomannan. Consistent with this hypothesis, it was reported that added sugars restricted the hydration of guar gum in the solution (Elfak *et al.*, 1977; Doyle *et al.*, 2006). The findings in the present study suggest that the inhibitory effect of galactomannan on the rate of glucose uptake may in part be due to the fully hydrated chains of galactomannan network.

To further study the effect of high concentration of sugar on hydration of galactomannan *in vivo*, we chose OGTT mixed with various concentrations of galactomannan solution to test in Sprague-Dawley rats. The results indicate that rats gavaged with 1% (w/w) galactomannan mixed with glucose load had significantly lower plasma glucose concentrations than rats gavaged with 0.5% (w/w) galactomannan mixed with glucose solution. These results suggest that 1% (w/w) galactomannan increased the viscosity of digesta more than the 0.5% (w/w) galactomannan. Thus the limitation of high galactomannan concentration mixed with high glucose concentration observed *in vitro* study (chapter 4) could be, at least, solved by the secretion in GIT leading to entanglement of fully hydrated chains of galactomannan. This finding is in agreement with the postprandial effect of viscous fibers, which were reported to increase the viscosity of digesta within the GIT (Nieuwenhoven *et al.*, 2001). This in turn is thought to increase the thickness of the UWL and slow diffusion transport of glucose toward the absorptive surface, and therefore attenuate the postprandial rise in blood glucose (Edwards and Read, 1990).

Based on these findings, it was further hypothesized that the viscosity of galactomannan extract may play important role on lowering plasma glucose, insulin, cholesterol, TG and adipose weight of rats. Therefore, feeding rats for a month with various concentrations of galactomannan mixed in diet was a logical next step.

Guar-gum ingestion has been reported to reduce insulin release; it appeared to be due mainly to lower glucose absorption (Jenkins *et al.*, 2000). In this study, rats fed with 5% (w/w) galactomannan-containing diet for 3 weeks confirmed that plasma insulin was significantly lower after OGTT although plasma glucose did not differ from the control. Moreover, rats fed with 5% (w/w) galactomannan-containing diet had significantly lower fasting insulin level. This suggests that feeding with 5% (w/w) galactomannan may improve the insulin sensitivity. This could be elucidated further by using insulin clamp technique. Rats fed with a diet containing 2.5% (w/w) galactomannan in this study did not reduce fasting plasma insulin compared to the control. This finding was consistent with other reports studying the effects of guar gum on plasma insulin; there was no change in plasma insulin concentrations during guar gum (10-20g/d) treatment compared to the control (Krotkiewski, 1984; Hagander *et al.*, 1984; Groop *et al.*, 1993).

Viscous fiber induces a feeling of satiety and thus reduces meal size and food intake (Mickelsen *et al.*, 1979; Porikos and Hagamen, 1986; French and Read, 1994). It was reported that consumption of fiber in a meal appears to reduce food ingested at a subsequent meal (Schwartz *et al.*, 1982; Stevens *et a*l., 1987). Feeding a diet containing 2.5 g guar gum reduced body weight by increased satiety, causing lower caloric intake (Kovac *et al.*, 2001). In our study, rats fed with 5% (w/w) galactomannan-containing diet ate less by 16% and weighed less by 25% compared to the control. The negative effects

on body weight and food intake did not occur in rats fed the 2.5% (w/w) galactomannan-containing diet. These observations suggest that feeding 2.5% (w/w) galactomannan-containing diet fed to growing rats (4 week old) did not have effect on food intake. While 5% (w/w) galactomannan-containing diet may have effect on reduced food appetite of rats.

The reduction of plasma cholesterol and hepatic cholesterol levels observed in this study was consistent with other reports demonstrating that increasing the viscosity of the intestinal contents was associated with reductions in both plasma and liver cholesterol concentrations (Marlett, 1997; Drzikova *et al.*, 2005). We did not determine the excretion of bile acids, nor cholesterol excretion in the current study. However, other studies of guar-galactomannan hypothesized the possible mechanism that increased luminal viscosity of galactomannan lowers cholesterol level by increased excretion of cholesterol and bile acids (Demigne *et al.*, 1998; Levrat *et al.*, 2000). Changes in enterohepatic cycling of bile acids are important in the regulation of bile acid production. Lowering bile acid pool increases the activity of HMG-CoA reductase, the rate limiting enzyme in the hepatic cholesterol synthesis pathway (Ast and Frishman 1990), and stimulates the activity of cholesterol 7 α-hydroxylase, the rate-limiting enzyme in bile acid synthesis (Lia *et al.*, 1995).

The lowering of plasma HDL-cholesterol observed in rats fed with 5% (w/w) galactomannan-containing diet in this study was consistent with significantly lowering of plasma LDL-cholesterol. In human, the LDL:HDL cholesterol ratio is a risk factor for CVD (Ascherio, 2006). LDL:HDL cholesterol ratio of this group was not significantly different from the control. The mechanism by which fiber lowers HDL cholesterol

remains undefined. However, the HDL-cholesterol lowering effect of 5% (w/w) galactomannan may not be suitable in cardiovascular disease treatment.

The reduction of plasma triglyceride and adipose triglyceride observed in the study suggests lower lipid absorption from the small intestine. It has been shown that viscous fibers impair lipid emulsification by increasing droplet size and hence decreasing the surface area (Pasquier *et al.*, 1996). The reduction in surface area is rate-limiting for lipolysis, since lipase can act only at the oil water interfaces (Pasquier *et al.*, 1996a). Increased luminal viscosities reduce convective diffusion and hence access of micelles to the brush border (Schiff *et al.*, 1972).

Unchanged FFA concentrations in rats fed 5% (w/w) galactomannan was seen in parallel with a significantly reduction in adipose triglyceride and epididymal fat pad weight. Based on these findings together with lowered food intake and altered epididymal histology, it was speculated that the lower energy intake may have stimulated lipolysis of the fat cells and increased FFA release into the circulation. However, to confirm this hypothesis one will have to include a pair-fed group in future experiments. Our results suggest that feeding 2.5% (w/w) galactomannan in the diet, significantly reduced epididymal fat pad weight with a decrease in FFA level. Therefore, the findings in this research represent a new aspect of adverse effect of feeding high dose of galactomannan (5%, w/w) to growing healthy rats (4 week old). In humans, there is not currently sufficient data to make evidence-based recommendations for a fiber requirement during childhood (Institute of medicine, 2005). The adequate intake (AI) for total fiber for children and adolescents is based on the data cited for adults, where 14g/1,000 kcal reduced the risk of CHD (Institute of medicine, 2005). Pediatricians have

been relatively cautious in recommending high-fiber foods for children (Committee on nutrition, 1981; Williams and Bollella, 1995). Objections have focused on concerns that high-fiber diets in childhood may compromise caloric intake and may compromise the bioavailability of minerals and other nutrients, such as riboflavin and protein.

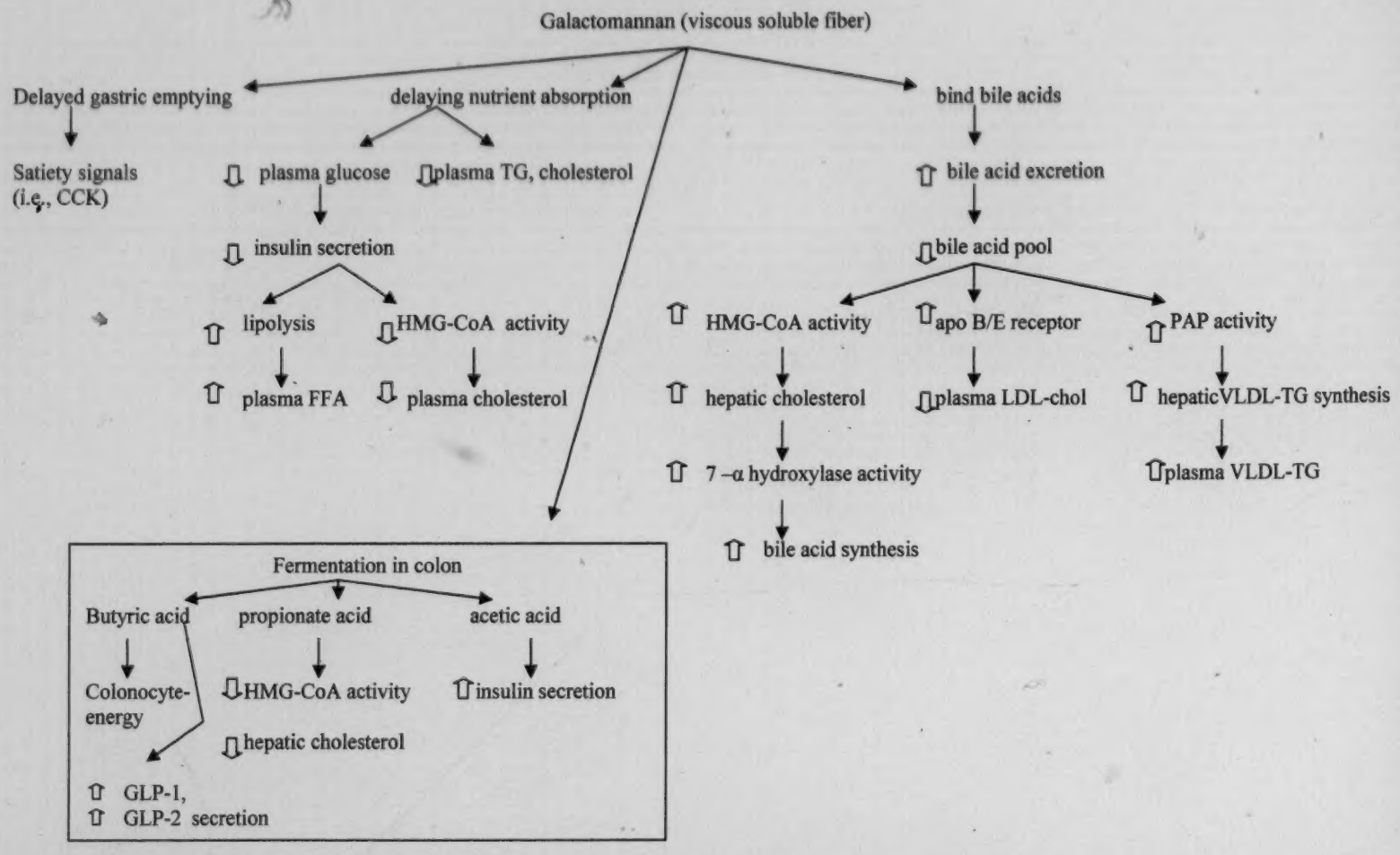
In future research, work needs to be done to further investigate the effect of galactomannan in obese animal models (i.e. Zucker diabetic fatty (ZDF) rats, Zucker fatty (*fa/fa)* rats, JCR (*cp/cp*) rats). A study on fructan, a fermented fiber, in growing obese Zucker fatty (*fa/fa)* rats reported that rats consuming 10% (w/w) fructan had a lower energy intake, a lower body weight and less TG accumulation in the liver when compared with the cellulose-control group (Daubioul *et al.*, 2002). Based on the results in this thesis and the literature, it would be interesting to investigate the effects of galactomannan extract on glycemic and lipid status in ZDF rats (consistently developed hyperglycemia model) feeding 2.5% and 5% (w/w) galactomannan in a high sucrose diet, compared to 10% (w/w) cellulose-containing high sucrose diet in the control group. The next study should be conducted over a 3 month period to determine long term effects of galactomannan, particularly on the metabolism in adipose tissue.

A more challenging project would be to study the contributory factors of high galactomannan-containing diet on the activity of hormone sensitive lipase (HSL), the rate-limiting enzyme in triglyceride breakdown (Holm *et al.*, 2000). When plasma insulin is decreased, HSL is activated to release more FFA into the circulation. (Institute of medicine, 2005). It is shown that FFA changes in plasma reflect changes of phosphorylation and translocation of HSL (Mook *et al.*, 2004). In adipose tissue, HSL is activated by hormones such as catecholamines, adrenocorticotropic hormone (ACTH),

and glucagons via cAMP-dependent protein kinase A (PKA) and inhibited by insulin (Degerman *et al.*, 1997; Holm *et al.*, 2000; Kraemer *et al.*, 2002). Since our results showed lower plasma insulin in rats fed with 5% (w/w) galactomannan containing diet along with high level of FFA, it is important to further investigate the activity of HSL in adipose tissue, including catecholamine level. In humans, catecholamines are the major activators of *in vivo* lipolysis, leading to phosphorylation of HSL through the induction of the cyclic adenosine monophosphate (cAMP)- dependent protein kinase, resulting in an elevation of plasma FFA levels (Clifford *et al.*, 2000; Tanset *et al.*, 2001; Martinez-Botas *et al.*, 2000).

The results of the current research are important contribution for the development of simple and cost effective extraction method for galactomannan from Amber fenugreek seeds. The method developed provides high purity and yield of galactomannan, and has high potential for scale-up production in the food industry. The viscosity and viscoelastic properties of galactomannan extract presents possibilities for its use in the food industry. Based on our findings in the *in vitro* and *in vivo* studies, galactomanann extract (when fed in levels up to 2.5% w/w in the diet) can be a safe alternative product for lowering plasma glucose, cholesterol, and triglyceride in preventive perspective. Our findings indicate the potential of galactomannan as a new source of dietary fiber that would increase the demand for Alberta grown fenugreek.

Figure 6-1 Hypothesized mechanisms of galactomannan to reduce plasma glucose and lipid



Abbreviation: PAP = phosphadic acid phosphatase activity, HMG-CoA= HMG-CoA reductase, CCK = Cholecystokinin

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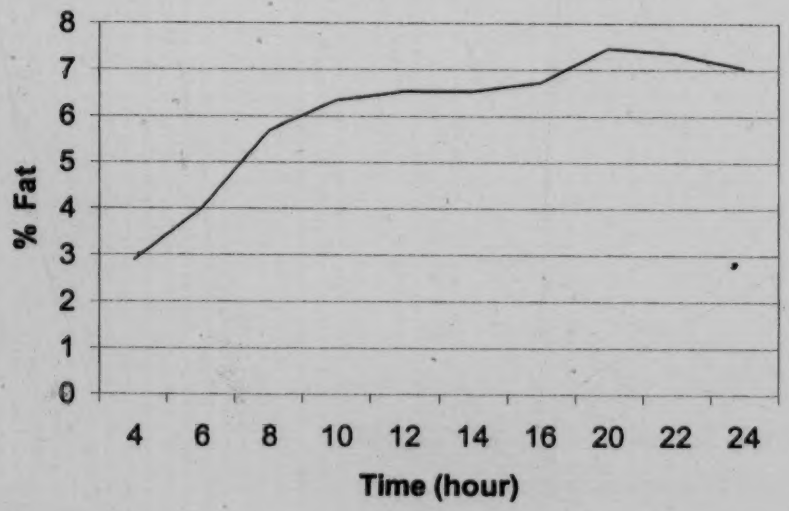
Appendix 1

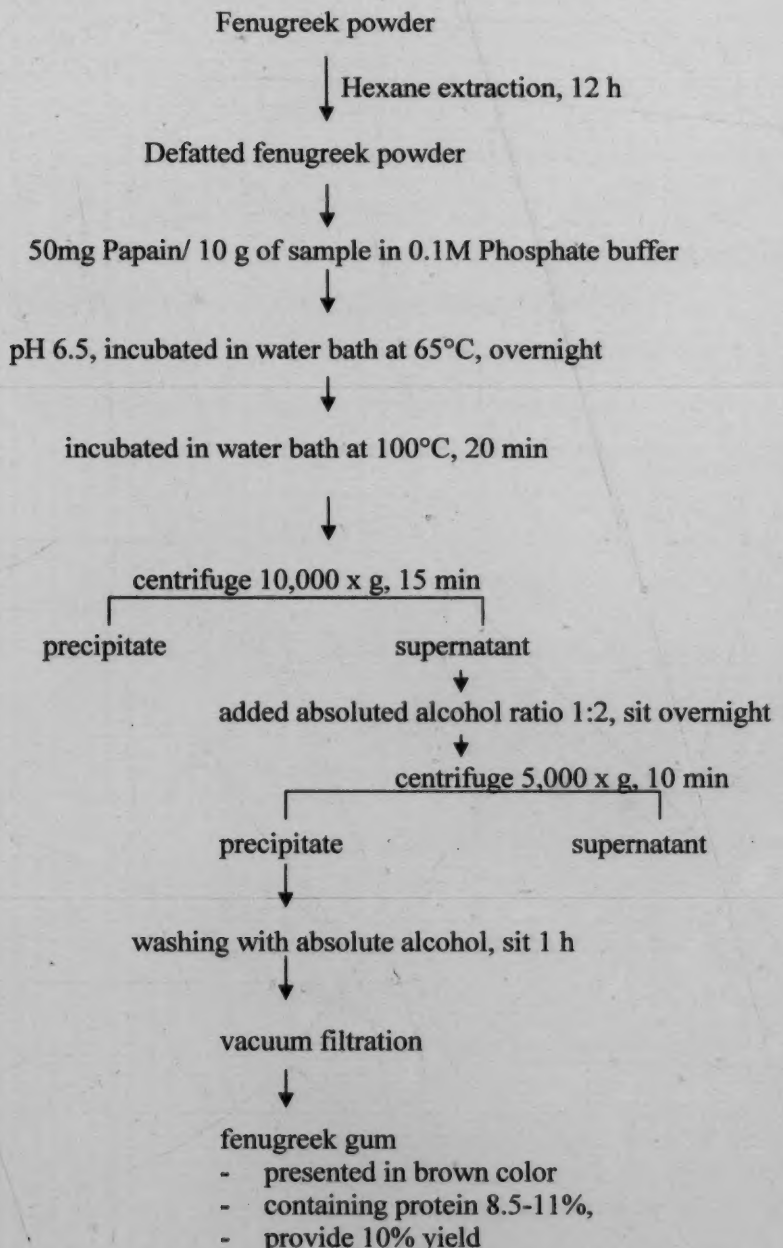
Hexane extraction of fenugreek seeds

The graph shows the amount of fat eliminated from fenugreek seeds in different hours. The graph shows the first plateau curve at 10 hours. At 20 hours, fat was mostly eliminated.



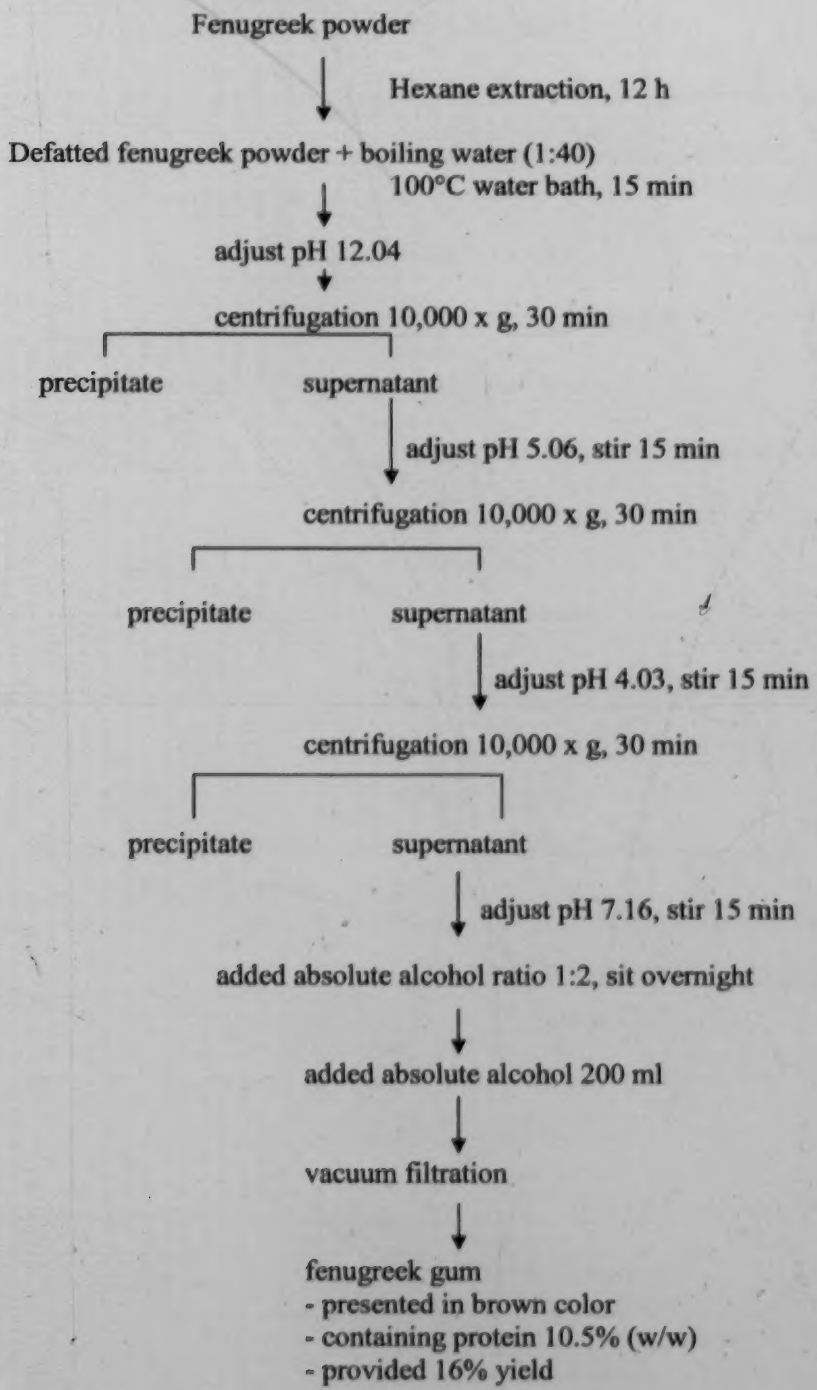
Appendix 2

Galactomannan extraction method using Papain®


Fenugreek powder
Hexane extraction, 12 h
Defatted fenugreek powder
50mg Papain/ 10 g of sample in 0.1M Phosphate buffer
pH 6.5, incubated in water bath at 65°C, overnight
incubated in water bath at 100°C, 20 min
centrifuge 10,000 x g, 15 min
precipitate
supernatant
added absoluted alcohol ratio 1:2, sit overnight
centrifuge 5,000 x g, 10 min
precipitate
supernatant
washing with absolute alcohol, sit 1 h
vacuum filtration
fenugreek gum
- presented in brown color
- containing protein 8.5-11%,
- provide 10% yield

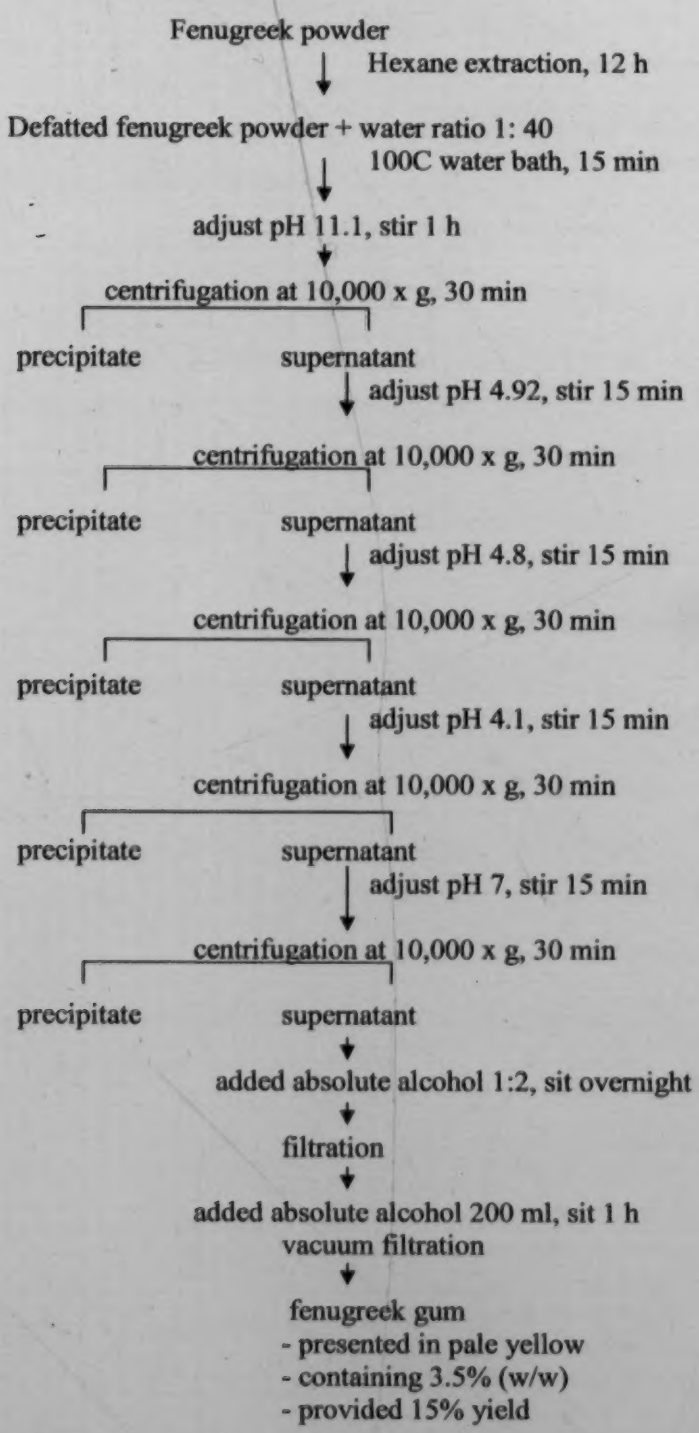
Appendix 3

Galactomannan extraction method using Na_2CO_3



Appendix 4

Galactomannan extraction method using NaOH



Appendix 5

Scanning electron microscopy (SEM) of a cross-section of fenugreek seeds of Lines Amber (A), F-70 (B), F-86 (C), L-3314 (D), and an Indian variety (E).

Appendix 6

Effect of each variable on glucose uptake in jejunum of both lean and obese JCR rats

Source	DF	Mean Square	p-value
Glucose level	1	4341526.691	< .0001
Galactomannnan	4	161944.585	< .0001
Genotype of rats	1	41884.157	0.1127
Glucose x galactomannan	4	29641.306	0.1308
Glucose x genotype of rats	1	12422.167	0.3869
Galactomannan x type of rats	4	22597.186	0.2460

DF = degree of freedom

Appendix 7

Effect of each variable on glucose uptake in ileum of both lean and obese JCR rats

Source	DF	Mean Square	p-value
Glucose level	1	8400132.818	< .0001
Galactomannnan	4	272056.877	< .0001
Genotype of rats	1	3755.707	0.7468
Glucose x galactomannan	4	118502.914	0.0117
Glucose x genotype of rats	1	174092.093	0.0286
Galactomannan x type of rats	4	26570.721	0.5661

DF = degree of freedom

Appendix 8

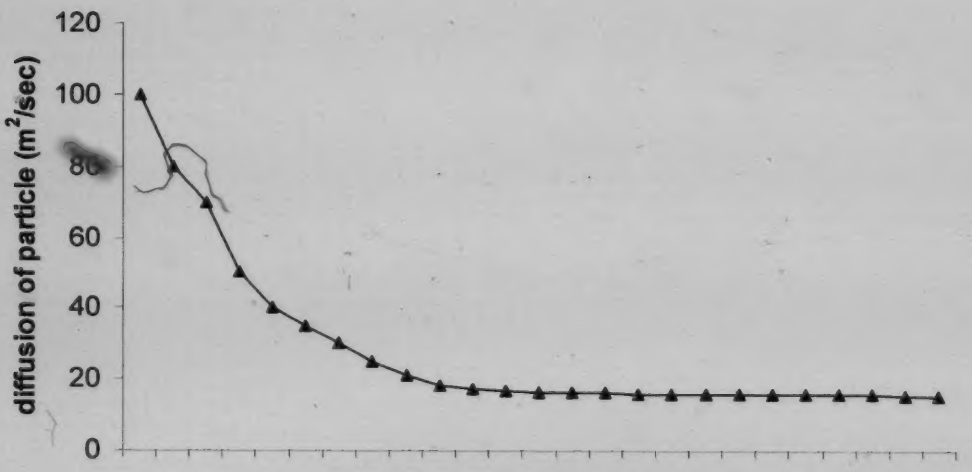
The diffusion coefficients in the liquid phase of solute (i.e. glucose)

Stokes-Einstein equation:

$$D = \frac{KT}{6 \P \eta r}$$

Where;
D is the Diffusion constant (m^2/sec)
K is Boltzmann's constant (J/K) or [(m^2 kg)/(s^2 K)]
T is the absolute temperature (Kelvin, K)
η is the fluid viscosity (kg/ second x meter)
r is the radius of the spherical solute (m)

The equation generally shows that the rate of diffusion constant is inversely proportional to the fluid viscosity (assuming that no change in *r*, *K* and *T* value). The diffusion is reduced when the viscosity is increased as shown in the diagram below. This graph was made based on the theory.



Appendix 9

Linear regression of glucose uptake rate in the jejunum tested in 2 mM glucose mixed with various galactomannan solutions

Source	DF	Sum of Square	Mean Square	p-value
Model	1	255095	255095	<0.0001
Error	128	941394	7354.639	
Corrected total	129	1196489		

DF = degree of freedom

Parameters	Value
Root MSE	85.75920
Dependent mean	92.69054
Coefficient mean	92.52206
R-Square	0.2132
Adjusted R-Square	0.2071

Variable	DF	Parameter estimate	Standard error	p-value
Intercept	1	143.15946	11.40219	<0.0001
Galactomannan	1	-240.76919	40.88186	<0.0001

DF = degree of freedom

Statistical model: Glucose uptake = 143.1 – 240.7 (X)

Where; X = galactomannan concentration

Appendix 10

Linear regression of glucose uptake rate in the ileum tested in 2 mM glucose mixed with various galactomannan solutions

Source	DF	Sum of Square	Mean Square	p-value
Model	1	443692	443692	<0.0001
Error	135	2794789	20702	
Corrected total	136	3238481		

DF = degree of freedom

Parameters	Value
Root MSE	143.88237
Dependent mean	161.63774
Coefficient mean	89.01534
R-Square	0.1370
Adjusted R-Square	0.1306

Variable	DF	Parameter estimate	Standard error	p-value
Intercept	1	226.29514	18.60568	<0.0001
Galactomannan	1	-308.64334	66.66893	<0.0001

DF = degree of freedom

Statistical model: Glucose uptake = 226.2 – 308.6 (X)

Where; X = galactomannan concentration

Appendix 11

Non-linear relationship of glucose uptake rate in the jejunum tested in 32 mM glucose mixed with various galactomannan concentrations

Source	DF	Sum of Square	Mean Square	p-value
Model	2	480848	240424	0.001
Error	137	3455646	25224	
Corrected total	139	3936494		

DF = degree of freedom

Parameters	Value
Root MSE	158.81969
Dependent mean	359.82386
Coefficient mean	44.13818
R-Square	0.1222
Adjusted R-Square	0.1093

Variable	DF	Parameter estimate	Standard error	p-value
Intercept	1	428.52455	22.21868	<0.0001
Galactomannan	1	-1083.25689	253.20900	<0.0001
Galactomannan^2	1	1994.25888	517.95871	0.0002

DF = degree of freedom

Statistical model: Glucose uptake = $428.5 - 1083.2\ (X) + 1994.2\ (X^2)$

Where; X = galactomannan concentration

Appendix 12

Non-linear relationship of glucose uptake rate in the ileum tested in 32 mM glucose mixed with various galactomannan concentrations

Source	DF	Sum of Square	Mean Square	p-value
Model	2	839851	419925	0.0005
Error	141	7330941	51992	
Corrected total	143	8170791		

DF = degree of freedom

Parameters	Value
Root MSE	228.01861
Dependent mean	526.94514
Coefficient mean	43.27179
R-Square	0.1028
Adjusted R-Square	0.0901

Variable	DF	Parameter estimate	Standard error	p-value
Intercept	1	605.20529	31.27970	<0.0001
Galactomannan	1	-1435.21754	357.12498	<0.0001
Galactomannan^2	1	2845.05634	734.15706	0.0002

DF = degree of freedom

Statistical model: Glucose uptake = $605.2 - 1435.2\ (X) + 2815\ (X^2)$

Where; X = galactomannan concentration

Appendix 13

Product development and sensory acceptability of fenugreek-loaf

I would like to thank Minh Huynh and Greg Rusnak for helping me to develop the fenugreek-loaf product.

Fenugreek seeds are normally used as a spice because of its strong sensory characteristics. However, the bitter saponins limit their acceptability in foods. The extraction of fat soluble saponins proved to be helpful, but the bitterness still remains. To overcome this obstacle, researchers tried to mask the bitterness by using chocolate and raspberry flavours. It is difficult to incorporate spice into food in large amounts because it takes only small amounts of the spice to unbearably overpower any other flavours in the product. To incorporate a strong bitter-spice like into a product, the latter has to have very pronounced flavours that could decrease the intensity of the fenugreek.

One recipe of the loaf that we developed has the following ingredients and their proportion.

Ingredient	Amount in grams
Margarine	41.7
Oil	42.0
White sugar	72.5
Brown sugar	72.5
Eggs	12.5
Buttermilk	42.0
Cocoa powder	41.9
Baking powder	0.83
Baking soda	1.67
Salt	1.67
Flour	208.3
Zucchini, grated	178.3
Raspberry jam	7.6
Almonds, coconut, chocolate chips	8.0 each
Defatted-fenugreek powder	15.0

The finished product was stored in the refrigerator at 4°C for 2 days before the sensory evaluation was carried out. On the day of the sensory evaluation date, the loaf was cut into rectangular pieces weighing approximately 50g/piece, and then warmed up in the microwave for 30 seconds to reach room temperature before serving. The panelists were presented with a piece of the product on white Styrofoam together a fork, and a questionnaire. The panelists were university students and staff. The product was served to 56 untrained panelists. Before the test, they were informed about the ingredients of the product, in case of allergies. They were also provided a consent form.

The affective testing method was used for the sensory evaluation. The 9-point Hedonic scale was chosen for measuring the categories of aroma, flavour and overall acceptability of the product. The numeric equivalent of the intervals is: 9= like extremely; 5=neither like nor dislike; 1=dislike extremely. The Just-about-right scale was used for the categories of moistness and aftertaste because we wanted to get an indication of the direction for future improvement of the product. The bipolar scale has five categories with 5=very (moist), 3= just about right, 1= very dry. The aftertaste scale is a little different with numeric values of: 5=very strong, 3=slightly weak, 1=no aftertaste. The characteristics of the product are presented as mean± SEM as shown below. The results showed that 80% of the panelists accepted the product despite a 'slightly weak' aftertaste.

Sensory characteristics of choco-zuchini fenugreek loaf in 56 panelists

Characteristics	mean ±SEM
Overall acceptability	7.14±0.19 (like moderately)
Aftertaste	3.13±0.13 (Just-about-right to slightly bitter)
Moistness	3.57±0.13 (Just-about-right to moist)
Aroma	7.05±0.17 (like moderately)
Flavor	7.18±0.20 (like moderately)

PUBLICATION AND PRESENTATIONS ARISING FROM THIS THESIS

The data presented in Chapter 2 has been accepted for publication:

Srichamroen A., Vasanthan T., Ooraikul B., Acharya SN., Basu TK. Dry-Fractionation improves the aqueous extractability and yield of galactomannans from fenugreek seeds. International Journal of Food Science and Nutrition.

Portions of research from this thesis have been presented as abstracts:

Srichamroen A., Vasanthan T., Ooraikul B., Basu TK. Isolation of galactomannan from fenugreek seeds. 31th International symposium on IFT, New Orleans, Louisiana, June 2004. (Poster Presentation)

Srichamroen A., Drozdowski L., Thomson ABR., Basu TK. The effect of galactomannan on glucose uptake in the intestine of insulin resistant JCR: LA-cp rats. ABS# 157, pg. 85. Canadian Diabetes association conference. Edmonton, Alberta, October 2005. (Poster Presentation)

Srichamroen A., Basu TK. The modifying effects of galactomannan of Alberta-grown fenugreek on glycemic and lipidemic status in rats. ABS# 17. The CSCN 5th Annual Scientific Meeting. Edmonton, Alberta, May 2006. (Poster Presentation)